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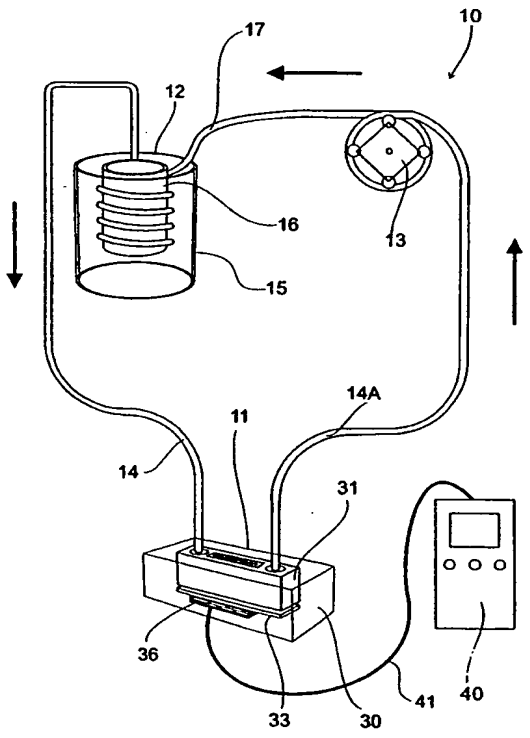
(54) Title: PROCESS FOR DETECTING POINT MUTATIONS IN WHICH THE PRIMER IS BOUND TO A SOLID SUPPORT AND EXTENSION DOES NOT OCCUR IF THE TERMINAL NUCLEOTIDE(S) MIS-MATCH

(57) Abstract

A process for determination of at least one target nucleotide at at least one particular location within at least one polynucleotide sequence in a test sample, said target nucleotide(s) constituting a 5' terminal nucleotide of a target nucleotide sequence within said polynucleotide sequence(s), said process including the steps of: (i) immobilizing one or more arrays of identical oligonucleotide primers with a solid support defining one or more reaction zones wherein each of said oligonucleotide primers in a respective array is capable of hybridizing with a corresponding target nucleotide sequence; (ii) contacting the reaction zone with the test sample; (iii) hybridizing at least one of said oligonucleotide primers with the polynucleotide sequence to form a hybrid wherein the polynucleotide sequence extends in a 3' to 5' direction beyond the 3' terminal nucleotide of the at least one oligonucleotide primer; (iv) extending the at least one oligonucleotide primer of the hybrid beyond the 3' terminal nucleotide thereof in the 5' to 3' direction using the polynucleotide sequence as a template in the case when the 3' terminal nucleotide of the at least one oligonucleotide primer is complementary to the 5' terminal nucleotide of said target nucleotide sequence, said extension effected in the presence of a polymerisation agent and nucleotide precursors to form a duplex including an extended primer molecule wherein at least one nucleotide incorporated into the extended primer molecule is a detectably-modified nucleotide; (v) denaturing the duplex to free the polynucleotide sequence from the extended primer molecule; (vi) carrying out steps (iii) to (v) one or more times; and (vii) detecting presence of a signal associated with the detectable-modified nucleotide in the extended primer molecule at the reaction zone to effect said determination. And an apparatus for PCR.



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TITLE

PROCESS FOR DETECTING POINT MUTATIONS IN WHICH THE PRIMER IS BOUND TO A SOLID SUPPORT AND EXTENSION DOES NOT OCCUR IF THE TERMINAL NUCLEOTIDE(S) MIS-MATCH

FIELD OF THE INVENTION

THIS INVENTION relates generally to nucleic acid sequence detection and in particular a process for determining a target nucleotide at a specific location within a polynucleotide sequence. The invention also relates to an apparatus for carrying out nucleic acid determinations inclusive of the process of the invention.

BACKGROUND OF THE INVENTION

A central theme in modern genetics is the relationship between genetic variability and phenotype. To this extent, a single nucleotide position on a strand of DNA may be responsible for polymorphism or allelic variation. Such variation within a coding sequence can result in a frame shift, a stop codon, or a non-conservative amino acid substitution, each of which can independently render a protein encoded by the coding sequence inactive.

Many genetic diseases and traits, for example, hemophilia, sickle-cell anaemia, cystic fibrosis and a variety of inborn errors of metabolism, reflect the consequences of variation at single nucleotide positions that have arisen in the genomes of some members of a species through mutation or evolution (Gusella, J.F., 1986, *Ann. Rev. Biochem.* **55** 831-854). In some cases, such 'polymorphisms' are linked to a genetic locus responsible for the disease or trait; in other cases, the polymorphisms are the determinative characteristic of the condition.

Several techniques have been developed to determine the presence or absence of specific variation at a single nucleotide position. Practical application of such techniques includes but is not limited to diagnosis of inherited or acquired genetic diseases, gene typing, karyotyping, genotyping, prenatal testing, parentage determination, DNA

family planning and forensic analysis.

Current detection procedures capable of detecting single nucleotide substitutions include procedures based on differential denaturation of mismatched probes, as in allele specific oligonucleotide hybridization (Wallace *et al.*, 1979, *Nucleic Acids Res.* **6** 3553) or denaturing gradient gel electrophoresis (Myers *et al.*, 1985, *Nature* **313** 495). While such techniques have the advantage of surveying long stretches of DNA for mismatched nucleotides, they are estimated to detect only about half of all mutations involving single nucleotides.

A commonly used method which may be utilized for screening DNA polymorphisms arising from DNA sequence variation consists of digesting DNA with restriction endonucleases and analysing the resulting fragments by means of Southern blots, as described by Botstein, *et al.* (1980, *Am. J. Hum. Genet.* **32** 314-331) and White *et al.* (1988, *Sci. Am.* **258** 40-48). Mutations in the DNA sequence that alter the endonuclease recognition sequence will preclude enzymatic cleavage at that site, thereby altering the cleavage pattern produced by such digestion. Subsequent to gel electrophoresis of the products of the digestion, DNA bands are compared between samples to detect differences in the pattern of restriction fragment lengths. A major problem with this method (known as Restriction Fragment Length Polymorphism analysis or RFLP analysis) is its inability to detect mutations that do not affect cleavage with a restriction endonuclease. In addition, RFLP analysis is typically reliant on gel electrophoresis and Southern blot analysis which are very labour intensive and time consuming techniques.

An alternative technique for detecting specific mutations in a segment of DNA is described in an article by Wallace *et al.* (1981, *Nucleic Acids Res.* **9** 879-894). This technique, known as Single Stranded Conformational Polymorphism (SSCP) analysis, involves hybridizing a DNA to be analysed (target DNA) with a complementary, labeled oligonucleotide probe. DNA duplexes which have one or more base pair

mismatches between the oligonucleotide probe and the target DNA will have different melting temperatures compared to DNA duplexes which have perfect complementarity therebetween. Accordingly, differential melting temperature between the strands of the DNA duplex can be used to distinguish target DNAs that are perfectly complementary to the oligonucleotide probe from target DNAs that differ by as little as a single nucleotide. This technique, however, suffers from similar disadvantages compared to RFLP analysis.

Recently, the Polymerase Chain Reaction (PCR) (Saiki *et al*, 1988, *Science*, **239** 487-491) has been utilized to amplify specific regions of DNA samples and analysis of nucleotide sequence variation has been achieved by sequence analysis of the resultant PCR product or by hybridisation with allele specific probes. This methodology employs sequential hybridization reactions of anti-parallel primers followed by enzymatic primer extension with a heat stable DNA polymerase to generate typically microgram quantities of DNA from diminutive amounts of starting material. PCR techniques, however, have certain drawbacks including the time and technical expertise required for determination of sequences from PCR amplified samples as well as a number of separate analyses being required to test for variation at more than one DNA locus.

Alternative primer extension based methods have been developed. For example, US Patent No 4,656,127 to Mundy discloses a method for determining the identity of a variant nucleotide within a polynucleotide sequence using a specialized exonuclease-resistant nucleotide derivative *e.g.*, an α-thionucleotide. In this method, a primer is constructed which is complementary to a region of the polynucleotide sequence which is substantially adjacent the variant nucleotide. The primer is then permitted to hybridize to this region and the resulting hybrid is subjected to primer extension in the presence of a DNA polymerase and nucleoside triphosphates, one of which is an α-thionucleotide. The hybrid is then digested using an exonuclease enzyme which cannot utilise

thio-derivatised DNA as a substrate for its nucleolytic action. If the target nucleotide in the polynucleotide sequence is complementary to the thionucleotide in the reaction, the resulting extended primer molecule will be of a characteristic size and resistant to the exonuclease. Hybrids without thio-derivatised DNA will be digested. After an appropriate enzyme digest to remove underivatised molecules, the thio-derivatised molecule can be detected by gel electrophoresis or other separation method.

Another technique which also utilises extension of a primer to identify a variant nucleotide within a polynucleotide sequence is disclosed in US Patent No 4,851,331 to Vary and Diamond. This method differs from that disclosed by Mundy in that the 3' terminal nucleotide of the primer is complementary to the variant nucleotide of interest. Primer extension is typically effected in solution in the presence of a DNA polymerase and nucleoside triphosphates, one of which is detectably-labeled. Extension of the primer is predicated on the primer forming a hybrid with the polynucleotide sequence including the 3' terminal nucleotide of the primer. Any mismatch between this 3' terminal nucleotide and the variant nucleotide is counterproductive to primer extension. Accordingly, the presence or absence of an extended primer molecule is indicative of the presence or absence of the variant nucleotide in the polynucleotide sequence.

The methods of Mundy and of Vary and Diamond have certain drawbacks. The method of Mundy is relatively insensitive and is therefore reliant on comparably large amounts of the polynucleotide sequence. It is also cumbersome due to the requirements of the second, different enzymatic system (*i.e.*, exonuclease) in which the non-derivatised hybrids are digested. The method of Vary is also reliant on relatively large amounts of the polynucleotide sequence for sensitivity. Moreover, it typically requires immobilization of the primer extension product after the hybridization and extension steps have been effected so

that any unincorporated labeled nucleoside triphosphates may be separated prior to the detection step. This step substantially prolongs the time required to effect the assay.

Reference also may be made to a method known as Genetic Bit Analysis or GBA™ which is disclosed in International Application Publication No WO 92/15712 (Goelet *et al.*). This method utilizes mixtures of labeled dideoxynucleoside triphosphate terminators and an oligonucleotide primer that is complementary to the sequence 3' to a polymorphic site. The labeled terminator that is incorporated on the primer by a DNA polymerase is thus determined by, and complementary to, the nucleotide present at the polymorphic site of the target molecule of interest. In a preferred embodiment, a heterogeneous phase assay is employed in which the primer or the target molecule is immobilized to a solid phase. Although the assay disclosed in Goelet *et al.* is easy to perform, this assay is comparatively insensitive because of the relatively low amount of label which may be incorporated on the primer.

An alternative strategy for detecting DNA polymorphisms, the Oligonucleotide Ligation Assay (OLA) (Landegren *et al.*, 1988, *Science* **241** 1077-1080) employs two oligonucleotides which are designed to be capable of hybridizing to abutting sequences of a single-stranded target nucleotide sequence. One of the oligonucleotides is biotinylated (biotinylated probe) and the other is detectably labeled (reporter probe) wherein the 3' nucleotide of the biotinylated probe or the 5' nucleotide of the reporter probe corresponds to site on the DNA being analysed for mutation. These oligonucleotides are then hybridised to the target DNA being analysed and if there is perfect complementarity, the enzyme T4 DNA ligase covalently joins the biotinylated probe and the reporter probe. However, if the probes and target are mismatched at their junction, a covalent bond is not formed between the probes. Capture of the biotinylated probe on immobilized streptavidin and analysis for covalently-linked reporters determine the nature of the probe-target

interaction (matched or mismatched). Although this method affords relative simplicity in comparison to previous methods, a disadvantage of this method is that it has relatively low sensitivity. In this regard, Nickerson *et al.* (1990, *Proc. Natl. Acad. Sci. USA* **87** 8923-8927) have described a nucleic acid detection assay that combines attributes of OLA and PCR. In this method, PCR is used to achieve the exponential amplification of target DNA, which is then detected using OLA. In addition to requiring multiple and separate processing steps, one disadvantage associated with such combination is that all the disadvantages associated with OLA and PCR are inherited.

A related but improved method to those referred to above is known as the Ligase Chain Reaction (LCR) which is disclosed in International Application Publication No WO89/09835 (Orgel, L.E.). This method involves a cyclic 2-step reaction consisting of a high temperature melting step in which double-stranded target DNA unwinds to become single-stranded and a cooling step in which two abutting complementary oligonucleotides are annealed to the single-stranded target molecule and are ligated together by a heat stable DNA ligase. The products of the ligation from one cycle serve as templates for the ligation products of the next cycle and repetition of such cycling results in an exponential amplification of template DNA in an analogous fashion to that of PCR. A disadvantage of this method, however, is that it is time consuming because of long incubation times required per cycle to effect ligation of adjacent oligonucleotides.

Having regard to the above, there is a continuing need for improvement in detecting variation at a single nucleotide position, particularly with regard to sensitivity, specificity, ease of use, and reduced assay time.

OBJECT OF THE INVENTION

Accordingly, it is an object of the present invention to provide a process for determination of a target nucleotide at a specific

location within a polynucleotide sequence which process may ameliorate at least some of the aforementioned disadvantages.

It is also an object of the invention to provide an apparatus for conducting nucleic acid determinations inclusive of the process of the invention.

SUMMARY OF THE INVENTION

According to one aspect of the present invention, there is provided a process for determination of at least one target nucleotide at at least one particular location within at least one polynucleotide sequence in a test sample, said target nucleotide(s) constituting a 5' terminal nucleotide of a target nucleotide sequence within said polynucleotide sequence(s), said process including the steps of:

(i) immobilizing one or more arrays of identical oligonucleotide primers with a solid support defining one or more reaction zones wherein each of said oligonucleotide primers in a respective array is capable of hybridizing with a corresponding target nucleotide sequence;

(ii) contacting the reaction zone with the test sample;

(iii) hybridizing at least one of said oligonucleotide primers with the polynucleotide sequence to form a hybrid wherein the polynucleotide sequence extends in a 3' to 5' direction beyond the 3' terminal nucleotide of the at least one oligonucleotide primer;

(iv) extending the at least one oligonucleotide primer of the hybrid beyond the 3' terminal nucleotide thereof in the 5' to 3' direction using the polynucleotide sequence as a template in the case when the 3' terminal nucleotide of the at least one oligonucleotide primer is complementary to the 5' terminal nucleotide of said target nucleotide sequence, said extension effected in the presence of a polymerization agent and nucleotide precursors to form a duplex including an extended primer molecule wherein at least one nucleotide incorporated into the extended primer molecule is a detectably-modified nucleotide;

(v) denaturing the duplex to free the polynucleotide

sequence from the extended primer molecule;

(vi) carrying out steps (iii) to (v) one or more times; and

(vii) detecting presence of a signal associated with the detectably-modified nucleotide in the extended primer molecule at the reaction zone to effect said determination.

For convenience, the above process of the invention will be referred to hereinafter as RASPE ("Recirculating Allele-Specific Primer Extension").

Throughout this specification and the appendant claims, unless the context requires otherwise, the words "comprise", "comprises" and "comprising" will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Suitably, the step of immobilizing (i) is characterized in that a single array of identical oligonucleotide primers is immobilized at a single reaction zone of the solid support.

Alternatively, the step of immobilizing (i) is characterized in that a plurality of distinct arrays of oligonucleotide primers are immobilized at discrete reaction zones of the solid support, wherein each of said reaction zones comprises a single array of identical oligonucleotide primers capable of determining a single target nucleotide under test.

The steps (ii)-(iii) may be conducted sequentially or simultaneously.

By "determination" is meant identifying a target nucleotide at a specific location or detecting the presence or absence of the target nucleotide.

The term "nucleotide sequence" or "polynucleotide sequence" as used herein designates mRNA, RNA, cRNA, cDNA or DNA. One of skill in the art will appreciate that cDNA is complementary DNA produced from a RNA template, usually by the action of RNA-dependent DNA polymerase ("reverse transcriptase").

The term "nucleotide" as used herein can refer to nucleotides present in either DNA or RNA and thus includes nucleotides which incorporate adenine, cytosine, guanine, thymine and uracil as base, the sugar moiety being deoxyribose or ribose. It will be appreciated however that other modified bases capable of base pairing with one of the conventional bases, adenine, cytosine, guanine, thymine and uracil may be used in the oligonucleotide primer employed in the invention. Such modified bases include, for example, inosine, 8-azaguanine and hypoxanthine.

By "nucleotide precursors" are meant any compounds which may be used by a polymerization agent to incorporate a nucleotide into a nucleic acid strand. Preferably, the nucleotide precursors are nucleoside triphosphates. Alternatively, the nucleotide precursors may comprise terminator nucleoside triphosphates such as, for example, dideoxynucleoside triphosphates (ddNTPs) which terminate chain elongation of the extended primer molecule. Suitable concentrations of ddNTPs for incorporation into the extended primer molecule may be readily determined by those skilled in the art.

The term "oligonucleotide" as used herein refers to a molecule comprised of two or preferably more than three deoxyribonucleotides or ribonucleotides, synthetic or natural. The exact size of the molecule may vary depending on the particular application.

The term "primer" as used in this specification refers to an oligonucleotide which, when paired with a strand of DNA, is capable of initiating the synthesis of a primer extension product in the presence of a suitable polymerizing agent. Preferably, the primer is an oligoribonucleotide and more preferably is an oligodeoxyribonucleotide. Alternatively, the primer may be other than a ribonucleotide. Suitably, the primer is sufficiently long to hybridize uniquely to the target nucleotide sequence. The exact length of the primer utilized in the method of the invention is dependent on a variety of factors, including the degree of

specificity of pairing required and the temperature and ionic strength during hybridization. Such factors are well known to those of skill in the art. In the case of peptide nucleic acid (PNA) oligonucleotide primers, for example, it is well known that the hybridization temperatures required to be employed to effect optimal hybridization between such oligonucleotides and a polynucleotide sequence will generally be higher than that for immobilized DNA oligonucleotides (Thiede *et al.*, 1996, *Nucleic Acids Res.* **24** 983-984, which is hereby incorporated by reference).

"Hybridization" is used here to denote the pairing of complementary nucleotide sequences to produce a DNA-DNA hybrid or a DNA-RNA hybrid. Complementary base sequences are those sequences that are related by the base-pairing rules. In DNA, A pairs with T and C pairs with G. In RNA U pairs with A and C pairs with G. In this regard, the terms "match" and "mismatch" as used herein refer to the hybridization potential of paired nucleotides in complementary nucleic acid strands. Matched nucleotides hybridize efficiently, such as the classical A-T and G-C base pair mentioned above. Mismatches are other combinations of nucleotides which do not hybridize efficiently.

Oligonucleotide primers may be selected to be "substantially complementary" to the target nucleotide sequence being tested. By "substantially complementary", it is meant that the oligonucleotide primer is sufficiently complementary to hybridize with a target nucleotide sequence. Accordingly, the nucleotide sequence of the oligonucleotide primer need not reflect the exact complementary sequence of the target nucleotide sequence. In a preferred embodiment, the oligonucleotide primer contains no mismatches with the target nucleotide sequence except, in certain instances, at or adjacent the 5' terminal nucleotide of the target nucleotide sequence. In this regard, when it is desired to detect the target nucleotide within the polynucleotide sequence (*i.e.*, the 5' terminal nucleotide of the target nucleotide sequence), it is generally preferred to

design the oligonucleotide primer to form a hybrid which includes the target nucleotide. The exact length of the oligonucleotide primer will depend on many factors including temperature and source of primer and use of the method. For example, depending upon the complexity of the target sequence, the oligonucleotide primer may typically contain 12 to 35 nucleotides, for example 15 to 30 nucleotides capable of hybridization to the target nucleotide sequence although it may contain more or fewer such nucleotides.

Oligonucleotide primers may be prepared using any suitable method, such as, for example, the phosphotriester method as described in an article by Narang *et al.* (1979, *Methods Enzymol.* **68** 90) and US Patent Specification No 4,356,270 which are hereby incorporated by reference. Alternatively, the phosphodiester method as described in Brown *et al.* (1979, *Methods Enzymol.* **68** 109) which is hereby incorporated by reference may be used for such preparation. Automated embodiments of the above methods may also be used. For example, in one such automated embodiment, diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucage *et al.* (1981, *Tetrahedron Letters* **22** 1859-1862) which is hereby incorporated by reference. Reference also may be made to United States Patent Specification Nos 4,458,066 and 4,500,707, which are hereby incorporated by reference, which refer to methods for synthesizing oligonucleotide primers on a modified solid support. It is also possible to use a primer which has been isolated from a biological source (such as a denatured strand of a restriction endonuclease digest of plasmid or phage DNA). In a preferred embodiment, the oligonucleotide primer is synthesized according to the method disclosed in United States Specification No 5,424,186 (Fodor *et al.*) which is hereby incorporated by reference. This method uses lithographic techniques to synthesize a plurality of different oligonucleotides at precisely known locations on a substrate surface.

In step (i) of the RASPE process, an oligonucleotide primer is immobilized to a solid support preferably at concentrations in excess relative to the concentration of the polynucleotide sequence. Such solid support may be comprised of natural, synthetic or naturally occurring materials which are synthetically modified including, but not limited to, cellulose materials such as paper, cellulose and cellulose derivatives such as cellulose acetate and nitrocellulose; glass or glass fibres; natural or synthetic cloth; plastics; nylon; porous gels such as agarose, silica gel, dextran and gelatin; porous fibrous matrixes; starch based materials such as Sephadex® cross-linked dextran chains; ceramic materials; latex; films of polyvinyl chloride and polyamide; polystyrene; polycarbonate; and combinations of polyvinyl chloride-silica and the like. In a preferred embodiment, the solid support is characterized in that it is substantially planar. For example, a two dimensional substrate as described in US 5,424,186 (Fodor *et al.*, *supra*) may be employed. Such substrate may be used to synthesize two dimensional spatially addressed oligonucleotide (matrix) arrays. Alternatively, the solid support may be characterized in that it forms a tubular array in which a two dimensional planar sheet is rolled into a three dimensional tubular configuration. Such configuration could be used to immobilize a greater surface area of oligonucleotide primers into a flow through cell as described hereinafter.

The oligonucleotide primer may be immobilized to the solid support using any suitable technique. For example, Holstrom *et al.* (1993, *Anal. Biochem.* **209**278-283), which is hereby incorporated by reference, exploit the affinity of biotin for avidin and streptavidin, and immobilize biotinylated nucleic acid molecules to avidin/streptavidin coated supports. Another method which may be employed involves precoating of polystyrene or glass solid phases with poly-L-Lys or poly-L-Lys, Phe, followed by covalent attachment of either amino- or sulfhydryl-modified oligonucleotides using bifunctional cross linking reagents (Running *et al.*, 1990, *Biotechniques* **8** 276-277; Newton *et al.*, 1993, *Nucleic Acids Res.*

21 1155-1162 which are hereby incorporated by reference). Kawai *et al.* (1993, *Anal. Biochem.* **209** 63-69) describe an alternative method in which short oligonucleotide probes are ligated to form multimers before cloning thereof into a phagemid vector. The oligonucleotides are then immobilized onto a polystyrene plate and fixed by U.V. irradiation at 254 nm. Reference also may be made to a method for the direct covalent attachment of short, 5'-phosphorylated oligonucleotide primers to chemically modified polystyrene plates ("Covalink" plate, Nunc) (Rasmussen *et al.*, 1991, *Anal. Biochem.* **198** 138-142, which is hereby incorporated by reference). Regard may also be had to an article by O'Connell-Maloney *et al.* (1996, *TIBTECH* **14** 401-407, which is hereby incorporated by reference) which discloses immobilization of biotinylated oligonucleotides and sulfhydrylated oligonucleotides respectively to a streptavidin-coated silicon wafer and an iodoacetamide-coated silicon wafer. Also, amino-modified oligonucleotides have been immobilized on isothiocyanate-coated glass (Guo *et al.*, 1994, *Nucleic Acids Res.* **22** 5456-5465, which is hereby incorporated by reference) and silane-epoxide-coated wafer (Eggers *et al.*, 1994, *BioTechniques* **17** 516-5240, which is hereby incorporated by reference). The aforementioned methods refer to post-synthetic attachment of oligonucleotide primers to a substrate. Alternatively, the oligonucleotide primers may be synthesized *in situ* utilizing, for example, the method of Maskos and Southern (1992, *Nucleic Acids Res.* **20** 1679-1684) or that of Fodor *et al.* (*supra*).

It will of course be appreciated that the oligonucleotide primers used in the invention may be immobilized either directly or indirectly. For example, the oligonucleotide primer may be covalently bound to a spacer molecule which has been covalently bound to the solid support. The spacer molecule may include a latex microparticle, a protein such as bovine serum albumin (BSA) or a polymer such as dextran or poly-(ethylene glycol). Such a spacer molecule is considered to improve accessibility of the oligonucleotide primer to hybridization of the target

nucleotide sequence. Alternatively, the spacer molecule may comprise an homo-polynucleotide tail such as, for example, oligo-dT. In a preferred embodiment, the spacer molecule may be 10 to 25 atoms in length.

A suitable test sample which may be used in the RASPE process may include extracts of double or single stranded nucleic acids obtained from prokaryotic or eukaryotic origin. For example, such extracts may be obtained from cells or tissue of plant or animal origin as well as cells derived from bacteria and viruses. By "obtained from" is meant that the nucleic acid extract is isolated from, or derived from, a particular source of the host. For example, the nucleic acid extract may be obtained from cells or tissue isolated directly from an animal host.

Sample extracts of DNA or RNA may be prepared following a cell lysis step which includes, but is not limited to, lysis effected by treatment with SDS, osmotic shock, guanidinium isothiocyanate and lysozyme. Suitable DNA which may be used in the RASPE process may include genomic DNA or cDNA. Such DNA may be prepared by any one of a number of commonly used protocols as for example described in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Ausubel, *et al.*, eds.) (John Wiley & Sons, Inc. 1995) and MOLECULAR CLONING. A LABORATORY MANUAL (Sambrook, *et al.*, eds.) (Cold Spring Harbor Press, 1989) which are hereby incorporated by reference. Sample extracts of RNA may be prepared by any suitable protocol as for example described in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (*supra*), MOLECULAR CLONING. A LABORATORY MANUAL (*supra*) and Chomczynski and Sacchi (1987, *Anal. Biochem.* **162** 156, hereby incorporated by reference).

The genomic DNA or cDNA may be fragmented, for example, by sonication or by treatment with restriction endonucleases. Suitably, the genomic DNA or cDNA is fragmented such that resultant DNA fragments are of a length greater than the length of the immobilized oligonucleotide primer(s) but small enough to allow rapid access thereto

under suitable hybridization conditions. Preferably, the length of polynucleotide sequence which is used as a template for extension of the oligonucleotide primer in step (iii) is at least 100 nucleotides. Alternatively, fragments of genomic DNA or cDNA may be amplified using a suitable nucleotide amplification technique. Such amplification techniques are well known to those of skill in the art and include, for example, PCR (Saiki *et al*, 1988, *supra*), Strand Displacement Amplification (SDA) (US 5,422,252, Little *et al.*), Rolling Circle Replication (RCR) (Liu *et al.*, 1996, *J. Am. Chem. Soc.* **118** 1587-1594; International Application Publication No WO 92/01813), Nucleic Acid Sequence Based Amplification (NASBA) (Sooknanan *et al.*, 1994, *Biotechniques* **17** 1077-1080) and Q-β replicase amplification (Tyagi *et al.*, 1996, *Proc. Natl. Acad. Sci. USA* **93** 5395-5400) all of which are hereby incorporated by reference.

The hybrid forming step (step (iii)), can be performed under suitable conditions for hybridizing oligonucleotide primers to test DNA or RNA which are well known to those of skill in the art. In this regard, reference may be made, for example, to NUCLEIC ACID HYBRIDIZATION, A PRACTICAL APPROACH (Homes and Higgins, eds.) (IRL press, Washington D.C., 1985). In general, whether such hybridization takes place is influenced by the length of the oligonucleotide primer and the polynucleotide sequence under test, the pH, the temperature, the concentration of mono- and divalent cations, the proportion of G and C nucleotides in the hybrid-forming region, the viscosity of the medium and the possible presence of denaturants. Such variables also influence the time required for hybridization. The preferred conditions will therefore depend upon the particular application. Such empirical conditions, however, can be routinely determined without undue experimentation.

The RASPE process is predicated on the condition that high efficiency enzyme-catalyzed extension of the oligonucleotide primer will

only proceed if there is complementarity between the 3' terminal nucleotide of the oligonucleotide primer and the 5' terminal nucleotide of the target nucleotide sequence. Accordingly, in the case of such complementarity, the oligonucleotide primer will be extended preferentially relative to an oligonucleotide primer with a mismatched 3' terminal nucleotide (Newton *et al.*, 1989, *Nucleic Acids Res.* **17** 2503-2516; Wu *et al.*, 1988, *Proc. Natl. Acad. Sci USA* **86** 2757-2760).

It will also be appreciated that if the polynucleotide sequence under test contains two strands (*e.g.*, genomic DNA) or has secondary structure which may preclude hybridization and/or primer extension of the oligonucleotide primer (*e.g.*, RNA), it is desirable to separate the strands of the polynucleotide sequence before it can be used as a template for hybridization and primer extension, either as a separate step or simultaneously with the synthesis of the extended primer molecule. This strand separation can be accomplished by any suitable denaturing method including physical, chemical or enzymatic means. One physical method of separating the strands of the polynucleotide sequence involves heating the polynucleotide sequence until it is substantially completely (>99%) denatured. Typical heat denaturation may involve temperatures ranging from about 80°C to 105°C for times ranging from about 1 to 10 minutes. Strand separation may also be induced by an enzyme from the class of enzymes known as helicases or the enzyme RecA, which has helicase activity and in the presence of riboATP (rATP) is known to denature DNA. Suitable reaction conditions for separating the strands of nucleic acids with helicases are described by Kuhn Hoffmann-Berling (1978, *CSH-Quantitative Biology* **43** 63), and techniques for using RecA are reviewed in Radding (1982, *Ann. Rev. Genetics* **16** 405-437). Alternatively, electrical denaturation may be used to effect denaturation of the polynucleotide sequence by, for example, applying low voltage electricity through the test sample (Purvis *et al.*, 1996, 4th World Congress on Biosensors, Bangkok. p 39, Elsevier

Advanced Technology, Oxford, which is hereby incorporated by reference), or by treating the test sample with dilute acid (*e.g.*, 0.25 M HCl for 7.5 to 10 minutes (Meinkoth and Wahl, 1984, *Anal. Biochem.* **138** 267-284).

After a hybrid is formed in the hybrid forming step (step (iii)), the oligonucleotide primer of the hybrid is subjected to primer extension by a polymerization agent. The polymerization agent may be a primer-dependent DNA polymerase or a viral reverse transcriptase. Such enzymes have the effect of incorporating nucleoside triphosphates into an extension of the oligonucleotide primer strand of the hybrid (*i.e.*, the extended primer molecule), either selectively for hybrids that contain perfectly matched pairings between the 3' terminal nucleotide of the oligonucleotide primer and the 5' terminal nucleotide of the target nucleotide sequence or non-selectively whether or not such pairing is perfectly matched. In this regard, it is well known that some enzymes such as, for example, Avian Myeloma Virus (AMV) reverse transcriptase and eukaryotic primer-dependent DNA polymerases have no 3' error-correcting activity (exonuclease$^-$ (exo$^-$)), and therefore will primer-extend only oligonucleotide primers which are bound in hybrids containing a perfect match between the 3' terminal nucleotide of the oligonucleotide primer and the 5' terminal nucleotide of the target nucleotide sequence. Alternatively, other polymerization agents, such as primer dependent DNA polymerases of prokaryotic origin including, for example, the "Klenow fragment" of *Escherichia coli* DNA polymerase I, have an error-correcting activity (exonuclease$^+$ (exo$^+$)), and therefore do not show this selectivity (although modifications of such enzymes are available from commercial suppliers wherein such error-correcting activity is absent). In this regard, enzymes such as Klenow may be utilized in the method of the invention because the exonuclease activity thereof is generally slower than elongation and thus not likely to occur in high frequency with the hybrid. However, it is preferable to employ 3' exo$^-$ polymerization agents for the

primer extension step. Suitable DNA polymerases which may be utilized in accordance with the present invention include, but are not limited to T7 DNA polymerase (exo$^-$) (*e.g.*, "Sequenase™" and "Thermosequenase™"), *Thermus aquaticus* (Taq) DNA polymerase, and Tth polymerase. Alternatively, strand displacing polymerases such as Vent™ (exo$^-$) polymerase may be used. Suitable reverse transcriptases which may be used in accordance with the invention include, but are not limited to, AMV reverse transcriptase and Moloney Murine Leukemia Virus (MMLV) reverse transcriptase and derivatives thereof. Preferably, the polymerizing agent is a thermostable enzyme which is stable to heat and is heat-resistant and catalyzes polymerization of nucleotides in the proper manner to form a primer extension product which is complementary with the template strand. Other factors in selecting the polymerization agent include: (a) whether the sample polynucleotide sequence is DNA or RNA (*e.g.*, only reverse transcriptases will effectively incorporate deoxynucleoside triphosphates into an extension product on an RNA template); (b) ability to incorporate detectably-modified nucleoside triphosphates (*e.g.*, biotin-UTP is not easily incorporated by AMV reverse transcriptase but is easily incorporated by Klenow fragment).

During the elongation step (step (iv)), all of the nucleotide precursors required for elongation should be present, with at least one of such precursors being a detectably-modified nucleotide precursor. The detectably-modified nucleotide precursor may have a label associated therewith which includes the following: 1. direct attachment of the label to the detectably-modified nucleotide precursor; 2. indirect attachment of the label to the detectably-modified nucleotide precursor; (*i.e.*, attachment of the label to a secondary intermediate which subsequently binds to the detectably-modified nucleotide precursor); and 3. attachment to a subsequent reaction product of the detectably-modified nucleotide precursor. Preferably, the label is attached directly to the detectably-modified nucleotide precursor. The label may be selected from a group

including a chromogen, a catalyst, an enzyme, a fluorophore, a luminescent molecule, a chemiluminescent molecule, a lanthanide ion such as Europium (Eu^{34}), a radioisotope and a direct visual label. In the case of a direct visual label, use may be made of a colloidal metallic or non-metallic particle, a dye particle, an enzyme or a substrate, an organic polymer, a latex particle, a liposome, or other vesicle containing a signal producing substance and the like. A large number of enzymes suitable for use as labels is disclosed in United States Patent Specifications U.S. 4,366,241, U.S. 4,843,000, and U.S. 4,849,338, all of which are herein incorporated by reference. Suitable enzyme labels useful in the present invention include alkaline phosphatase, horseradish peroxidase, luciferase, β-galactosidase, glucose oxidase, lysozyme, malate dehydrogenase and the like. The enzyme label may be used alone or in combination with a second enzyme which is in solution. Alternatively, a fluorophore which may be used as a suitable label in accordance with the present invention includes, but is not limited to, fluorescein, rhodamine, Texas red, lucifer yellow or R-phycoerythrin. It will also be appreciated that, in the case of indirect attachment of the label (2.), reagents such as biotin, digoxigenin, streptavidin and various protein antigens act as "bridges" and require the presence of a second intermediate for production of a detectable signal. For biotin, the secondary intermediate may include streptavidin enzyme conjugates. For antigen labels, secondary intermediates may include antibody-enzyme conjugates.

In step (v), it is necessary to denature or separate the extended primer molecule from the polynucleotide sequence to permit the polynucleotide sequence to hybridize in step (iii) to another of the oligonucleotide primers for extension thereof. Such denaturation or separation may be effected using any suitable conditions as, for example, referred to above.

The steps of hybrid formation (step (iii)), primer extension (step (iv)) and denaturation (step (v)) may be repeated as often as

needed to ensure that a substantial number of detectably-modified nucleotide precursors participate in the reaction and so that the signal produced by detectably-modified nucleotides in the extended primer molecule is detectable by a suitable detection means. In this regard, the signal generated in the reaction zone may be detected by visual inspection or by an instrumental means. Preferably, the signal is detected by the instrumental means. In such a case, the signal is preferably generated by a direct visual label such as a dye particle and the preferred method of detection is by visual detection, for example, by use of a photocell and scanning light beam or a charge coupled device (CCD) in concert with an immobile light source. Depending on the nature of the label, a signal may be instrumentally detected by irradiating a fluorescent label with light and detecting fluorescence in a fluorimeter; by providing for an enzyme system to produce a dye which could be detected using a spectrophotometer; or detection of a dye particle or a colored colloidal metallic or non metallic particle using a reflectometer; in the case of using a radioactive label, luminescent molecule or chemiluminescent molecule employing a radiation counter or autoradiography.

Subsequent to a final primer extension step, it is preferred to perform a separation step to remove substantially any unincorporated detectably-modified nucleotide precursors which may be a potential source of unwanted background. Such separation may be effected, for example, by utilizing a washing step.

In step (vii), the presence or absence of detectably-modified nucleotide(s) in the extended primer molecule is determinative of the presence or absence of an allelic complementation. Accordingly, if detectably-modified nucleotide(s) is incorporated in the extended primer molecule, then it will be detected in a unique location (*i.e.*, reaction zone) on the solid support indicating the presence of the target nucleotide at the particular location in the polynucleotide sequence. Conversely, if detectably-modified nucleotide(s) is not incorporated, then it will not be

detected in the reaction zone indicating the absence of the target nucleotide. Thus, the presence or absence of a target nucleotide corresponding with a specific allelic sequence may be determined by the presence or absence of detectably-modified nucleotide(s) in the reaction zone.

The RASPE process can be performed in a step-wise fashion where after each step new reagents are added, or simultaneously, where all the reagents are added at the initial step, or partially step-wise and partially simultaneous, where fresh reagent is added after a given number of steps. If a method of strand separation (step of denaturation, step (v)), such as heat, is employed which will inactivate the polymerization agent, as in the case of a heat-labile enzyme, then it is necessary to replenish the agent for polymerization after every strand separation. The simultaneous method may be utilized when a number of purified components, including an enzymatic means such as helicase, is used for the strand separation step. In the simultaneous procedure, the reaction mixture may contain, in addition to the polynucleotide sequence, the strand separating enzyme (*e.g.*, helicase), an appropriate energy source for the strand-separating enzyme, such as rATP, all the nucleotide precursors required for elongation, the immobilized oligonucleotide primer in molar excess and the polymerization agent, *e.g.*, Klenow fragment. If heat is used for denaturation in a simultaneous process, a heat stable polymerization agent such as Taq DNA polymerase, Tth polymerase or Vent™ (exo$^-$) polymerase, is preferably employed which will operate at an elevated temperature, preferably, 55°C to 90°C at which temperature the polynucleotide sequence will consist of single and double strands in equilibrium.

A plurality of target nucleotides corresponding to nucleotide variations may be determined using the above process. For example, pairs of oligonucleotide primers in discrete reaction zones may be used to hybridize respectively with a mutant allele and a wild-type allele.

Accordingly, the 3' terminal nucleotide of the first primer will be complementary to the 5' terminal nucleotide of the target nucleotide sequence corresponding to the mutant allele and the 3' terminal nucleotide of the second primer will be complementary to the 5' terminal nucleotide of the target nucleotide sequence corresponding to the wild-type allele. On the basis that allelic variation is due to a single nucleotide substitution corresponding to the 5' terminal nucleotide of the target sequences (or is correlated with such a substitution), the polynucleotide sequence under test can be treated using such oligonucleotide primers to determine whether an individual is homozygous or heterozygous with respect to those alleles. In this format, mutant alleles would show a negative response with the oligonucleotide primer specific for the wild-type allele, but give a positive response with their complementary oligonucleotide primer. If desired, the present invention may apply to the detection of up to three nucleotide substitutions corresponding to the 5' terminal nucleotide of a target nucleotide sequence.

In another embodiment, distinct arrays of oligonucleotide primers may be employed wherein each of the primers is complementary to a genetic locus of interest. Accordingly, the present invention may be applied, for example, to the diagnosis of β-thalassaemias in which a test sample may contain as many as 60, for example 50, separate potential variant sequences and therefore the test sample would contain as many as 60 target nucleotide sequences all of which may, if desired, be amplified and the products distinguished according to the present invention. Alternatively, the different oligonucleotide primers may be complementary to target nucleotide sequences associated with different inherited or acquired genetic disorders.

It will be appreciated that, in addition to directly detecting point mutations within a structural gene, the RASPE process also may be used to indirectly detect the presence of normal or defective structural genes. Thus, for example, various DNA sequence polymorphisms

genetically linked to the structural gene for known genetic defects are known to be highly correlated with that particular defect. Thus, in the case of sickle cell anemia, a linked *Hpa*I restriction endonuclease polymorphism has been found to be highly correlated with the β^S defect (Kan *et al.*, 1978, *Proc. Natl. Acad. Sci. USA* **75** 5631). In this case, an appropriate oligonucleotide primer may be designed and synthesized to detect the presence of the polymorphic *Hpa*I restriction site thereby permitting detection of a marker linked to the β^S allele. The indirect determination of a gene defect is most practical when a particular polymorphism is highly correlated with a defective phenotype. Thus, linked markers are useful, for example, when the defect is not known or when there are many defective alleles (*e.g.*, Lesch-Nyhans disease). In either case, it may be possible to follow the inheritance of these disorders and the prenatal diagnosis thereof, if a known polymorphism is associated with a particular defect which may be detected by the method of the invention.

According to another aspect of the invention, there is provided a solid support for determination of at least one target nucleotide at at least one particular location within at least one polynucleotide sequence in a test sample, said target nucleotide(s) constituting a 5' terminal nucleotide of a target nucleotide sequence within said polynucleotide sequence(s), said solid support comprising:

one or more arrays of identical oligonucleotide primers immobilized thereon defining one or more reaction zones, wherein each of said oligonucleotide primers in a respective array is capable of hybridizing with a corresponding target nucleotide sequence and has a 3' terminal nucleotide complementary to said target nucleotide to form a hybrid which permits extension of the oligonucleotide primer of the hybrid in the presence of a polymerization agent and nucleotide precursors.

In a further aspect, the invention resides in a kit for determination of at least one target nucleotide at at least one particular

location within at least one polynucleotide sequence in a test sample, said target nucleotide(s) constituting a 5' terminal nucleotide of a target nucleotide sequence within said polynucleotide sequence(s), said kit comprising:

(a) one or more arrays of identical oligonucleotide primers immobilized on a solid support defining one or more reaction zones, wherein each of said oligonucleotide primers in a respective array is capable of hybridizing with a corresponding target nucleotide sequence and has a 3' terminal nucleotide complementary to said target nucleotide to form a hybrid which permits extension of the oligonucleotide primer of the hybrid in the presence of a polymerization agent and nucleotide precursors;

(b) a plurality of nucleotide precursors, wherein at least one of said nucleotide precursors is a detectably-modified nucleotide precursor;

(c) a polymerization agent capable of extending said oliginucleotide primers in a 5' to 3' direction when the 3' terminal nucleotide of the oligonucleotide primer is complementary to the target nucleotide.

In yet another aspect of the invention, there is provided an apparatus for amplification and/or determination of a target nucleic acid, said apparatus comprising:

a reaction zone, in use, receiving a solid support having immobilized thereto first nucleic acids as well as receiving a reaction fluid for immersing therein said first nucleic acids, said reaction fluid comprising second nucleic acids being free in solution, wherein said target nucleic acid is included in said first nucleic acids or in said second nucleic acids;

a thermoregulation means for modulating the temperature of said reaction fluid at said reaction zone to facilitate at least in part said amplification and/or determination;

a denaturation zone for receiving said reaction fluid, and for

denaturing said second nucleic acids, said denaturation zone being spaced from said reaction zone and in fluid communication therewith; and

means for moving said reaction fluid between said reaction zone and said denaturation zone during the course of said amplification and/or determination.

A reaction zone according to the invention may have any suitable dimensions appropriate for accommodating said solid support and a volume of said reaction fluid sufficient to immerse the nucleic acids attached to the solid support. The reaction zone may be suitably located within a reaction chamber. In such a case, internal surfaces of the reaction chamber are preferably comprised of a hydrophobic material so that the reaction fluid does not adhere to said internal surfaces in use. The internal surfaces, in this regard, may be formed of any suitable hydrophobic material including, but not limited to, silicon, polycarbonate, teflon, polyvinylchloride and silanized glass. Preferably, the reaction chamber comprises means for retaining said solid support therein, and more preferably means for retaining said solid support at said reaction zone. The reaction chamber suitably has means for introducing the solid support and the reaction fluid therein prior to the commencement of said amplification and/or determination and for removing the solid support and the reaction fluid therefrom subsequent to the completion of said amplification and/or determination.

Preferably, the cross section of said reaction zone ensures a large heat exchange surface to volume ratio, and therefore enables rapid temperature variations of said reaction fluid.

The thermoregulation means may comprise any suitable heat exchanger for modulating the temperature of the reaction fluid at the reaction zone such that the reaction fluid may be conditioned rapidly to a temperature advantageous for effecting a step of the amplification/determination. The modulation of the reaction fluid temperature by said thermoregulation means may be effected by

convection, conduction, radiation or a combination of any two or more of these. Suitably, said thermoregulation means comprises a Peltier cell which may effect heating and cooling of the reaction fluid as desired. Preferably, said thermoregulation means is microprocessor controlled to effect automatic sequential modulation of the reaction fluid temperature at the reaction zone. Alternatively, the thermoregulation means may comprise a water bath which is set at a particular temperature.

Preferably, said thermoregulation means modulates the temperature of the reaction zone such that said zone is capable of conditioning the temperature of the reaction fluid at a level suitable for at least one of the following:

(i) hybridization of a probe nucleic acid to a target nucleic acid to form a hybrid, wherein said probe nucleic acid is substantially complementary to at least a portion of said target nucleic acid;

(ii) extension of the probe nucleic acid of the hybrid, in the presence a polymerization agent to form a duplex including an extended probe molecule; and

(iii) denaturation of the hybrid of (i) and/or the duplex of (ii) to separate the strands thereof.

The denaturation zone may effect denaturation of the second nucleic acids by any suitable means. For example, the denaturation zone may employ any of the methods of denaturation herein described.

Any suitable means may be employed to effect movement of the reaction fluid between the reaction zone and the denaturation zone. For example, a fluid transfer means may be used which preferably comprises at least one pump such as a vacuum pump or a peristaltic pump. Alternatively, the fluid transfer means may rely on a pressure differential between the reaction zone and the denaturation zone and may therefore include a pair of gas supply tubes.

Suitably, the apparatus further comprises one or more auxiliary vessels in fluid communication with the apparatus such that reagents for said amplification and/or determination may enter or exit the apparatus. Such reagents may include, for example, the second nucleic acids, dNTPs, ddNTP's, polymerization agents, buffers and washing solutions.

Preferably, the apparatus further comprises a display means for displaying the temperature of the reaction zone.

The apparatus preferably further comprises a detection means for detecting a probe nucleic acid or an extension product thereof attached to the solid support. Depending on the nature of a label associated with the detector nucleotide sequence or an extension product thereof, a signal may be instrumentally detected by irradiating a fluorescent label with light and detecting fluorescence in a fluorimeter; by providing for an enzyme system to produce a dye which could be detected using a spectrophotometer; or detection of a dye particle or a colored colloidal metallic or non metallic particle using a reflectometer; in the case of using a radioactive label or chemiluminescent molecule employing a radiation counter or autoradiography. Accordingly, the detection means may be adapted to detect or scan light associated with the label which light may include fluorescent, luminescent, focussed beam or laser light. In such a case, the detector nucleotide sequence or extension product thereof is preferably mounted on a charge couple device (CCD) or on a photocell and subsequently scanned for emission of light therefrom. In some cases, electronic detection of the signal may not be necessary. For example, with enzymatically generated colour spots associated with an oligonucleotide array format as herein described, visual examination of the array will allow interpretation of the pattern on the array. However, even for a simple array, the number of possible patterns is large (*e.g.*, for a 4 X 4 array there exists 2^{16} patterns). Thus, for such an example, the detection means is preferably interfaced with

pattern recognition software to convert the pattern of signals from the oligonucleotide array into a plain language genetic profile.

The apparatus of the invention may be utilized for carrying any suitable method which requires thermoregulation of a reaction and recirculation of denatured probe nucleic acid or denatured target nucleic acid. Such apparatus is particularly suited for carrying out the processes herein disclosed which may be effected according to automatic or non-automatic procedures.

In the present apparatus, the first nucleic acids attached to the solid support may comprise (1) the target nucleic acid or (2) the detector nucleic acid. With reference to (1), it will be appreciated that nucleic acid determinations which may be facilitated therewith include, but are not limited to, colony hybridizations, Southern hybridizations, and Northern hybridizations. Thus, for example, the apparatus of the invention may be utilized for carrying any suitable nucleic acid determination method which requires thermoregulation of a reaction and recirculation of denatured probe nucleic acid or denatured target nucleic acid. Accordingly, the subject apparatus provides a number of advantages over conventional forms of apparatus for nucleic acid determination. For example, in a conventional hybridization assay such as a Southern hybridization, a probe nucleic acid (probe) typically in double-stranded form is denatured to separate its strands so that the strands are available for hybridization to a target nucleic acid immobilized on a solid support. During the hybridization process however there is a propensity for these strands to reanneal or rehybridize to complementary probe strands thereby limiting availability of the single-stranded probe for hybridization to the target nucleic acid. The apparatus of the present invention may overcome this limitation because it frequently denatures a double-stranded probe to provide predominantly single-stranded probe which is circulated continuously over an immobilized target nucleic acid at the reaction zone. Denaturation of the double-stranded probe is effected

during a hybridization process in the denaturation zone which is in fluid communication with the reaction zone. The continuous flow of predominantly single-stranded probe over the immobilized target nucleic acid is considered to increase the rate at which the single-stranded probe locates, and hybridizes to, the target nucleic acid relative to conventional hybridization assays. This effectively increases the speed and sensitivity of such assays.

In the case of (2), suitable nucleic acid amplifications and/or determinations which may be facilitated therewith include, but are not limited to, the aforementioned RASPE process, and solid-state embodiments of the PCR process which are well known to those of ordinary skill in the art. With respect to the RASPE process, single-stranded probe nucleic acid may be immobilized to a solid support at the reaction zone (instead of the target nucleic acid), and the target nucleic acid may be frequently denatured (instead of the probe) at the denaturation zone to provide predominantly single-stranded target nucleic acid which is circulated continuously over the immobilized probe. The immobilized probe may be immersed in a reaction fluid at the reaction zone to facilitate the RASPE process and the temperature of the reaction fluid is suitably modulated by the thermoregulation means to carry out a number of sequential steps of the process consisting of hybridization, extension and denaturation. The apparatus, in this embodiment, is considered to decrease the effective time required to effect a denaturation step of the RASPE process because the denaturation zone provides the reaction zone with predominantly single-stranded target nucleic acid independently of that generated at the reaction zone during a denaturation step of the process. Moreover, the continuous flow of predominantly single-stranded target nucleic acid over the immobilized probe during a hybridization step of the RASPE process is also considered to increase the rate at which the single-stranded target locates, and hybridizes to, the immobilized probe. Thus, the subject

apparatus may increase the rate at which the RASPE process is facilitated relative to prior art thermal cyclers.

The above apparatus may also be used for solid-state embodiments of PCR such as, for example, the solid phase amplification process disclosed in International Application Publication No WO 93/09250, hereby incorporated by reference) by using a second single-stranded probe. This second probe may be either free in the reaction fluid or immobilized to the same solid support as the single-stranded probe used in the RASPE process. The second probe anneals to the target nucleic acid downstream of, and on the opposite strand to which, the first nucleic acid probe anneals. Thus, the first and second probes constitute conventional anti-parallel primers which are necessary for a PCR process.

In a further aspect of the invention, there is provided a process for amplification and/or determination of a target nucleic acid in a test sample suspected of containing said target nucleic acid, said method including the steps of:

(a) immobilizing first nucleic acids onto a solid support at a reaction zone;

(b) immersing said first nucleic acids in a reaction fluid comprising second nucleic acids being free in solution, wherein said target nucleic acid is included in said first nucleic acids or in said second nucleic acids if said target nucleic acid is present in said test sample;

(c) modulating the temperature of said reaction fluid at said reaction zone;

(d) denaturing said second nucleic acids at a denaturation zone being spaced from said reaction zone and in fluid communication therewith; and

(e) moving said reaction fluid between said reaction zone and said denaturation zone;

characterized in that said amplification and/or determination is effected after carrying out steps (c), (d) and (e) one or more times.

BRIEF DESCRIPTION OF THE DRAWINGS

In order that the invention may be readily understood and put into practical effect, particular preferred embodiments will now be described by way of example with reference to the accompanying drawings in which:

FIG. 1 is a graph showing the relationship between reaction zone signal intensity and the number of cycles of the RASPE process;

FIG. 2 is a photograph of three Immobilon® membranes showing reproducibility of signal generated by 20 cycles of the RASPE process with similar amounts of immobilized oligonucleotide primer.

FIG. 3 is a graph showing the influence of target concentration on the RASPE process;

FIG. 4 is a photograph showing that p53 target DNA specifically reacts with an oligonucleotide specific therefor;

FIG. 5 is a graph showing that the RASPE process discriminates between wild type and mutant alleles of the CFTR gene;

FIG. 6 is a perspective view of one embodiment of the apparatus according to the invention;

FIG. 7 is a cut-away perspective view of a reaction vessel relating to the apparatus of FIG. 6;

FIG. 8 is a cut-away perspective view of the reaction vessel illustrated in FIG. 6 showing an inert membrane being introduced thereto;

FIG. 9 is a cut-away perspective view of the reaction vessel illustrated in FIG. 6 showing an inert membrane placed within a reaction chamber of the reaction vessel;

FIG. 10 is a longitudinal cross sectional view of the apparatus of FIG. 8;

FIG. 11 is a transverse cross sectional view of the apparatus of FIG. 8;

FIG. 12 is a longitudinal cross sectional view of the apparatus of FIG. 9;

FIG. 13 is a transverse cross sectional view of the apparatus of FIG. 9; and

FIG. 14 is an alternate embodiment of the apparatus according to the invention.

EXAMPLE 1

Preparation of a RASPE reaction mix

In one example of the RASPE process, the following ingredients were used to prepare a reaction mix.

i) 100 µL Sequenase buffer made up as follows:

20 µL	5 x Sequenase buffer
10 µL	5% Tween 20/NP40
70 µL	distilled water

wherein 5 X Sequenase buffer comprises 200 mM Tris-HCl, pH 7.5 100 mM $MgCl_2$, 250 mM NaCl;

ii) 4.5 µL fluorescein nucleotide mix made up as follows:

0.5 µL	0.1 M DTT (dithiothreitol)
0.5 µL	0.1 M $MnCl_2$, 0.15 M NaIsocitrate
0.325 µL	5% Tween 20/NP40
0.375 µL	cold TAG (83 µM each of ddT, A, G)
0.25 µL	10 µM fluorescein ddCTP
2.55 µL	deionized distilled water (DDW)
2.0 µL	Sequenase diluted 1:26 in enzyme dilution buffer

wherein enzyme dilution buffer comprises 10 mM Tris-HCl, 5 mM DTT, 0.5 mg/mL Bovine Serum Albumin.

Preparation of a solid support

A biotinylated 40 mer allele-specific oligonucleotide primer, 5'-TTTCCTGGATTATGCCTGGCACCATTAAAGAAAATATCAT-3',

complementary to part of exon 10 of the cystic fibrosis transmembrane conductance regulator gene was coupled to a solid support as follows: 2.3 mg of streptavidin (Sigma cat. no. S4762) was dissolved in 2.3 mL of a 0.5 M potassium phosphate solution. Ten 8 x 8 mm squares ("wafers") of Millipore Immobilon® (activated PVDF) membrane (Cat No. IASD00005) were dropped into the Streptavidin solution and shaken at 150 rpm for 19 hr at 25°C. Spare sites on the membrane were capped with glycine. This has the additional advantage of adding a net negative charge to the membrane surface, thereby reducing non-specific binding of nucleic acids.

Capping solution was made as follows: to a 1 M solution of $NaHCO_3$, glycine was added to a concentration of 2 M and the pH adjusted to 9.6. For the capping process, the streptavidin solution was drained off the Immobilon® wafers. Six millilitres of glycine solution was added, shaken for a few seconds then drained off. Six millilitres of fresh glycine solution was added to the wafers and these were incubated with shaking at 150 rpm for 2 hr at 25°C. Wafers were then washed by rinsing once in 10 mL of PBS/0.1% Tween 20, then washing in two changes of PBS/0.1% Tween 20 for approximately 15 min per wash. Wafers were then drained and air dried on a paper tissue for 10 min then stored in a sealed bottle with silica gel desiccant at room temperature.

A stock solution of oligonucleotide was prepared in water at 150 pmol/µL. A volume of this solution was diluted with an equal volume of PBS/Tween 20, pH 7 for preparation of the membrane. Six microlitres of the diluted stock solution was spotted onto each wafer using a pipette. The wafers were incubated for 30 min at 25°C in a closed glass vial. Wafers were washed three times in PBS/Tween, with draining between washes, then air dried for 10 min and stored desiccated in a sealed glass bottle.

Alternatively, an oligonucleotide having an amino group at its 5' end may be attached directly onto the Immobilon® membrane and spare sites capped with 2 M glycine solution according to the

manufacturer's instructions which are hereby incorporated by reference.

Note that the number of different oligonucleotide spots on a strip is limited only by the spotting technology used. Machines (for example the BioDot dispense system, Bio Dot incorporated , Irvine, California) are available to spot oligonucleotides at less than 100 micron spacing with as little as 8 nanolitre drops. Alternatively arrays of 200 micron spots can be microjet printed onto a UV silica glass wafer at 800 micron spacing (Eggers et al. (1994), BioTechniques 17, page 516, hereby incorporated by reference).

RASPE reaction

One hundred microlitres of RASPE reaction mix was added to a reaction tube. The polynucleotide sequence under test was 0.23 picomoles of a 491 base pair fragment of exon 10 which was obtained by PCR amplification of a cloned CFTR gene sequence using the following primers: 5'-CATTCACAGTAGCTTACCCA-3' and 5'-GCAGAGTACCTGAAACAGGA-3'.

The polynucleotide sequence was added to 100 µL of Sequenase buffer and heated for 10 min at 95°C in a heat block to denature the DNA strands. Two oligonucleotide coated wafers were then immersed in the hot 1 x Sequenase buffer (~95°C) and allowed to cool to 37°C for 5 min. Sequenase and fluorescently labeled ddCTP and unlabeled ddNTPs were subsequently added and the primer extension reaction was allowed to proceed for 5 min. The reaction tube was then placed in a heat block set at 37°C. Cycling parameters were: 5 min at 95°C in one heat block; followed by 15 min at 37°C in another heat block to allow for hybridization of complementary strands and extension thereof. Sequenase enzyme (2 µL enzyme diluted 1:26 in enzyme dilution buffer) was added to the reaction fluid after each cycle. In an alternate embodiment as in Example 2-5, a thermostable DNA polymerase such as Thermosequenase™ or Taq DNA polymerase may be employed which obviates the need for the addition of enzyme after each cycle.

The process was repeated through 5 cycles and one wafer was removed and washed in phosphate buffered saline (PBS)/Tween for the colorimetric detection. A further five cycles were carried out on the remaining wafer, before it too was removed and washed for the detection step. A glycine coated wafer was used in the same process as a negative control.

The detection step entailed incubation of the wafer with an antibody to fluorescein. The antibody employed was conjugated to alkaline phosphatase. The wafer was washed, and then a substrate, nitroblue tetrazolium chloride (NBT) in dimethyl formamide (Boehringer Mannheim cat#1383213), was added subsequently. Alkaline phosphatase was then used to catalyze a colour change (clear to brown) where fluoresceinated ddCTP has been incorporated into the extended primer molecule. The density of color deposition was proportional to the number of cycles (FIG. 1). Since incorporation of dideoxynucleotides in the extended primer molecule prevents further extension of the primer molecule, the increase in color deposition per cycle reflects that different oligonucleotide primers are extended in each cycle. Accordingly, it can be concluded that the number of extended oligonucleotide primer molecules increases proportionally with the number of cycles.

In an alternative embodiment, detection was carried out by dropping the wafers (that had been reacted with the antibody-alkaline phosphatase conjugate) into a soluble substrate and measuring the change in absorbance of the solution at 405 nm (data not shown). Additional control experiments were performed to show that the increased incorporation of fluoresceinated ddCTP was not due to the longer total hybridization time, but rather due to the cyclical re-use of the polynucleotide sequence in repeat rounds of denaturation and extension.

The extension times and denaturation times used in the above experiments serve as illustrations of the process only and may be reduced substantially if desired, for example to between about 1 and 2

min.

EXAMPLE 2

In this example, oligonucleotides were directly coupled to Immobilon® strips via a 5'-amino group on the oligonucleotides. The oligonucleotide was a 23 mer with a 7 carbon linker at the 5' end as attached by the manufacturers (Gibco BRL). The sequence of the oligonucleotide (5'-NH_2-7 carbon linker-GTGGTAATCTACTGGGACGGAAC-3') which is complementary to part of exon 8 of the human p53 tumor suppressor gene. The oligonucleotide was purified by HPLC. Approximately 12 picomoles of this oligonucleotide was immobilized at each of three sites on the strips in a volume of 0.1 µL.

The reaction mix contained 25 µL of 0. 1 M Dithiothreitol (final concentration of 5 mM), 20 µL of 1 M Tris-HCl (final concentration 40 mM), 100 µL of 25 mM $MgCl_2$ (final concentration 5 mM), 250 µL 0.1 M NaCl (final concentration 50 mM), 10 µL each of dTTP, dGTP and dCTP to a final concentration of 50 µM, 10 µL of fluorescein-12-dATP (New England Nuclear Life Science Products, Boston, MA) to a final concentration of 2 µM, 25 µL of 10 mg/ml BSA, 20 µL DDW.

Target DNA (149 base pair segment of p53 exon 8, 0.2 picomoles) was added to the reaction chamber of the apparatus described in Example 6. The reaction mix was subjected to 20 cycles of RASPE in this apparatus, each cycle consisting of approximately 5 minutes at 95°C and 5 minutes at 37°C. Washing and developing of the strip was as described previously.

Densitometric analysis (not shown) of the color deposition on the strips presented in FIG. 2 indicates that identical amounts of oligonucleotide covalently attached to the Immobilon® strips give reproducible signal densities.

EXAMPLE 3

To demonstrate the influence of target concentration on the RASPE process, different amounts of p53 exon 8 target DNA (0.1 fmole

and 0.5 fmole) were reacted with an Immobilon® strip coated with 40 pmole of 5'-NH_2-7 carbon linker-GTGGTAATCTACTGGGACGGAAC-3' (prepared by Gibco BRL), an oligonucleotide complementary to a target sequence within p53 exon 8. The reaction mix comprised 100 µL 10 X buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, 0.01 %gelatin), 60 µL each cold dTTP, dCTP, dGTP (2.5 mM), 40 µL Fluorescein-12- dATP (New England BioLabs), 5 µL (25 Units) AmpliTaq™ polymerase (Perkin-Elmer), 33 µL Target DNA (p-53 Exon 8 diluted to give 0.1 fmole or 0.5 fmole per assay), 642 µL DDW. The reaction conditions were 40 cycles of: 5 min at 95°C followed by 5 min at 50°C. Washing and detection was carried out as described above except that the substrate for the colour development reaction was NBT/BCIP (nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt) (Boehringer Mannheim cat. no.1681451). The strip was submerged in 5 mL equilibration buffer with 100 µL NBT/BCIP for 8 minutes until color spots developed on strip. The strip was briefly air dried , then scanned on a Hewlett Packard Desk Scanner (ScanJet IIcx), then densitometry was carried out using the Molecular Dynamics (Sunnyvale,CA) Image Quant software.

The results, presented in FIG. 3, show that the color density increases as a function of the concentration of target DNA. Accordingly, by use of samples containing known concentrations of target nucleic acids as standards, the RASPE process can be used to quantify a target nucleic acid in a test sample. This feature is advantageous for assessing homozygosity or heterozygosity associated with a particular allele.

EXAMPLE 4

In another embodiment, 800 pmole of the following oligonucleotides were covalently linked to Immobilon® strips:

p-53 5'-NH_2-7 carbon linker-GTGGTAATCTACTGGGACGGAAC-3'

'CF' or 'CFWT'	5'-NH_2-7 carbon linker-AAAAAAAATTCATCATAGGAA ACACCAAA-3'
CFDEL	5'-NH_2-7 carbon linker-AAAAAAAATTAAAGAAAATAT CATTGG-3'
CF542T	5'-NH_2-7 carbon linker-TTTTTTTTTAAGACAATATAG TTCTTT -3'

Oligonucleotide CFWT is complementary along its entire sequence to a target sequence within wild type exon 10 of the CFTR gene. Oligonucleotide CFDEL is specific for a mutant CFTR exon 10 (which has a deletion of three bases). Oligonucleotide CF542T is specific for a mutant CFTR exon 11 (relating to codon 542 of the deduced CFTR polypeptide sequence).

The above oligonucleotide arrays were reacted with 0.2 pmole p53 exon 8 target DNA. The reaction conditions were the same as for Example 2 except that 30 cycles of the RASPE process were employed.

The results, presented in FIG. 4, indicate that the p53 target DNA reacted specifically with the immobilized p-53 oligonucleotide defined above. No cross reactivity was detected with the other oligonucleotides. Moreover, in another experiment in which CFTR target DNA was reacted under similar with immobilized p-53 oligonucleotide conditions, no cross reactivity was observed (results not shown). Accordingly, the RASPE process may be used to discriminate between various target sequences.

EXAMPLE 5

The RASPE process was used to discriminate between wild type and mutant alleles of the CFTR gene. Wild type CFWT and mutant CFDEL oligonucleotides each defined above were covalently attached to an Immobilon® membrane according to Example 4. The 3' nucleotide of the CFWT oligonucleotide is complementary to the 5' nucleotide of the

target sequence in wild type CF exon 10. By contrast, the last base of the CFDEL oligonucleotide does not match the wild type sequence and is therefore an inefficient primer for enzyme-catalyzed addition of nucleotides. The reaction mix was taken through 70 cycles of the RASPE process, each cycle consisting of 5 min at 95°C and 5 min at 50°C.

The results, shown in FIG. 5, indicate that the CFWT oligonucleotide is substantially preferentially extended by the RASPE process relative to the CFDEL oligonucleotide. Thus, the process of the invention can be used to discriminate between wild type and mutant alleles.

EXAMPLE 5

Referring now to FIGS. 6-13, there is shown an embodiment of the apparatus according to the invention. The apparatus shown generally at 10 is suitable for carrying out nucleic acid determinations inclusive of the RASPE process, PCR and conventional hybridization assays. The apparatus 10 comprises a reaction vessel 11, in fluid communication with a denaturation vessel 12 wherein the fluid communication is in closed circuit relationship. A peristaltic pump 13 is also provided which circulates a reaction fluid between the reaction vessel 11 and denaturation vessel 12 in a direction shown by the arrows.

Reaction vessel 11, shown in more detail in FIGS. 7-13, comprises an outer insulating shell 30 constructed of polystyrene, an intermediate sleeve 31 constructed of high density silicone rubber and a releasably connectable resilient central sleeve 32 constructed of a low density silicone rubber. In another embodiment, the intermediate sleeve 31 and the central sleeve 32 may be of a unitary construction.

Reaction vessel 11 also includes a glass slide 33 upon which an inert membrane 34 is positioned for nucleic acid determination (see FIG. 9). The inert membrane 34 may have immobilized thereon either arrays of oligonucleotide primers (*e.g.*, in the case of the RASPE process or PCR) or target nucleic acids (*e.g.*, in the case of Southern or

Northern blots).

Beneath an undersurface 32A of inner sleeve 32 and above an upper surface 33A of slide 33 (more clearly viewed in FIGS 10-13), there is defined a reaction chamber 35 for accommodating the inert membrane 34 and a reaction fluid therewithin.

A Peltier cell 36 is located beneath and in bearing contact with slide 33 for modulating the temperature thereof and the reaction fluid. A control unit 40 comprising a microprocessor is also provided which effects control of the temperature of the Peltier cell through lead 41.

Central sleeve 32 is releasably connectable with vessel 11 to allow removal of the reaction fluid from the reaction chamber 35 and/or introduction of the inert membrane 34 therewithin and/or removal therefrom. Central sleeve 32 when connected to intermediate sleeve 31 provides a substantially fluid tight seal which effectively inhibits escape of the reaction fluid from the reaction chamber 35. Alternatively, the inert membrane 34 may be introduced into reaction chamber 35 by way of slit 37 formed centrally of central slit 32. As can be seen more clearly in FIGS. 8-18, the slit 37 may be prised apart by use, for instance, of a pair of forceps and the membrane 34 guided between lips 37A of the slit 37 into chamber 35 where it is positioned on slide 33 as shown. Apart from being used to introduce the membrane 34 into the chamber 35, the slit 33, when closed, functions to substantially inhibit evaporation of the reaction fluid.

The reaction chamber 35 is in fluid communication with the pump 13 and the denaturation vessel 12 by way of silastic tubing 14 connected to inlet port 38 and outlet port 39. In the present embodiment the tubing 14 has an internal diameter of 0.76 mm.

The denaturation vessel 12 comprises a glass beaker 15, containing a fluid such as water at a temperature of 95°C to 100°C. Placed within the fluid of the beaker is a perspex cylinder 16 having tubing 14 coiled therearound. Optionally, a lid (not shown) may be provided

which is adapted to inhibit evaporation of the fluid from the container. In operation, the denaturation vessel 12 effects denaturation of the target nucleic acid or probe nucleic acid for nucleic acid determination at the reaction chamber 35.

A length of tubing 14 between denaturation vessel 12 and reaction vessel 11 is chosen such that the temperature of the reaction fluid entering reaction chamber 35 from denaturation vessel 12 does not decrease below the temperature of the reaction fluid required for the nucleic acid determination in reaction chamber 35.

The apparatus 10 further comprises a sample injection port 17 intermediate the pump 13 and denaturation vessel 12. In this embodiment, the injection port 15 may be utilized to inject into the apparatus 10 a sample of target nucleic acid or probe nucleic acid including the reaction fluid.

In operation, the membrane 34 is positioned on slide 33 with the immobilized nucleic acids facing the undersurface 32A of inner sleeve 32. Reaction fluid is then delivered into apparatus 10 through sample injection port 17. The volume of reaction fluid delivered therein may be up to the fluid capacity of apparatus 10 which, in this embodiment, is approximately 600 µL. However, this is not necessary since reaction fluid volumes of between 300 µL and 550 µL/min also work effectively.

The temperature of the reaction fluid in the reaction chamber 35 is modulated by control unit 40 to effect the nucleic acid determination. Fluid is then displaced along the fluid circuit of apparatus 10 by pump 13 at a rate of between 50 µL and 500 µL. The rate is selected such that nucleic acids in the reaction fluid are substantially denatured by denaturation vessel 12 before exiting therefrom and such that the reaction chamber 35 is provided with a continuous supply of predominantly single-stranded nucleic acids.

EXAMPLE 6

The apparatus 10 may be advantageously used for carrying

out automated Southern or Northern blots. According to one embodiment, the reaction chamber 35 comprises a membrane 34 (eg. a nylon membrane) on which is immobilized target nucleic acid (DNA or RNA is transferred to and immobilized the membrane by commonly known methods (Ausubel *et al.*, *supra*)). Southern blots are commonly carried out using a double stranded DNA probe that is complementary to denatured target DNA immobilized on the membrane 34. A preliminary step in conventional Southern blot detection is denaturation of the double stranded DNA probe to separate it into single strands which are capable of binding to the denatured target DNA.

A problem with conventional Southern blotting is that the complementary strands of the probe DNA tend to re-hybridize with each other in a reaction that competes with hybridization to single strand target DNA molecules immobilized on the membrane. This results in a reduced sensitivity of the process. The apparatus 10 is capable of facilitating probing of Southern blots with cyclical re-use of a double stranded DNA probe on the same immobilized target DNA. The process involves the following steps:

1) Approximately 500 µL of a pre-hybridization mix (*e.g.*, 0.01 M $NaPO_4$, 1 mM EDTA, 6 x SSC , 0.5% SDS, 2 x Denhardts solution, 100 μg/mL denatured salmon sperm DNA) is added to chamber 35 via sample injection port 17 to immerse the membrane 34. The Peltier cell 36 is set to maintain chamber 35 at a constant operating temperature of about 65°C.

2) After about 4 hours, the prehybridization solution is removed and fresh prehybridization solution is added to chamber 35. Chamber 35 remains heated at 65°C.

3) A double stranded DNA probe is labeled by a method such as random priming, which method is well known to those skilled in the art, with radioactively labeled nucleotides, digoxigenin labeled nucleotides or fluorescently labeled nucleotides. The double stranded

probe is denatured by heating at 95°C for 5 min and the resultant single-stranded DNA probe is added subsequently to chamber 35 through sample injection port 37 and operation of pump 13.

4) At the reaction chamber 35, the single stranded DNA probe hybridizes to the immobilized target DNA and any unreacted probe (*i.e.*, unhybridized) is recirculated through the denaturation vessel 12, where it is denatured again before flowing back into the reaction chamber 35 for further rounds of hybridization.

5) After hybridization, the hybridization reaction mix is removed by disconnecting the tube 14A from port 39 and flushing the chamber 35 with wash buffer (2 x SSC plus 0.1% SDS) through sample injection port 17 and subsequently reconnecting tube 14A to port 39. removing central sleeve 32 from the reaction vessel 11. Wash solutions with increased stringencies are then injected through port 17 and circulated over membrane 34 for about 20 min at about 65°C. Usually a series of wash steps is undertaken, with a change of wash buffer between each wash step.

6) The membrane 34 is then removed from vessel 11 by removal of central sleeve 32 therefrom and labeled probe attached to the target DNA (if target DNA is present on the membrane) is detected according to conventional detection procedures.

The above apparatus 10 allows more efficient use of labeled probe in that problems associated with probe re-annealing to its complementary strand instead of the target nucleic acid are substantially ameliorated.

EXAMPLE 7

Now turning to FIG. 14, there is described an alternate embodiment of the apparatus according to the invention. The apparatus generally shown at 100 is constructed of perspex or glass or other suitable material such as, for example a plastics material, and comprises a frusto-conical shaped denaturation chamber 101 in fluid communication

with a similarly-shaped reaction chamber 102 through channel 103.

In the reaction chamber there is disposed an Immobilon® membrane 104 comprising a plurality of distinct reaction zones 105 on which is immobilized a plurality of different allele-specific oligonucleotide primers. Shaded zones 106, in this embodiment, correspond to positive detection of specific target nucleic acids after carrying out the RASPE process.

Apparatus 100 further comprises ports 107 and 108 through which sample and/or reagents may be delivered into and/or out of apparatus 100.

In operation, a sample of polynucleotide sequence is delivered into the denaturation chamber 101 through port 107. This port 107 is subsequently closed and apparatus 100 inverted before immersion of the denaturation chamber 101 into a boiling water bath. After 5 min incubation therein, the apparatus 100 is inverted to drain the heat denatured sample from the denaturation chamber 101 into the reaction chamber 102. Any target nucleotide sequences complementary with the allele specific oligonucleotide primers will hybridize thereto and primer extension will proceed, for example at about room temperature, if Sequenase™ is used as the polymerization agent.

After an appropriate time of primer extension, the reaction chamber 102 is immersed in a boiling water bath to denature the polynucleotide sequence and any extended primer molecules that may have formed. The apparatus 100 is then inverted to drain the sample back into chamber 101. The reaction chamber 102 is then allowed to cool to about room temperature. The chamber 101 is again immersed in boiling water for 2 to 3 minutes, the apparatus is inverted and the denatured sample runs into chamber 102 for the next cycle of hybridization and extension. Cycles of heat denaturation in the boiling water bath and incubations at room temperature are repeated for a predetermined number of times to effect signal detection.

The process and apparatus of the invention accommodate simple, rapid, sensitive and automatable detection of variant nucleotides in polynucleotide sequences. The subject invention may be employed generally to detect allelic variation or polymorphism due to a single base substitution in a polynucleotide sequence. Such single nucleotide variation is known to be responsible for particular disease states such as acquired or inherited genetic diseases which may include cystic fibrosis, β-thalassaemia, haemophilia, sickle cell anaemia, and familial type III hypercholesterolaemia, Factor V Leiden and others readily apparent to those skilled in the art. The present invention may also be utilized for forensic investigations, genotyping, gene typing, karyotyping, DNA tissue typing or generally any situation in which there is nucleotide variation at a specific location in a polynucleotide sequence.

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The present invention has been described in terms of particular embodiments found or proposed by the present inventors to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scope of the invention. All such modifications and changes are intended to be included within the scope of the appended claims.

FIGURE LEGENDS

FIG. 1. A 40 mer allele-specific oligonucleotide primer complementary to the CFTR gene was immobilized on an Immobilon (PVDF) wafer and exposed to a 491 bp segment of Exon 10 of the CFTR gene (~0.24 picomole) for the designated number of cycles. "Control" wafer was coated with glycine only. Sequenase™ was replenished at the end of each cycle.

FIG. 2. 12 pmole of p53-specific oligonucleotide was immobilized at three different locations respectively on two Immobilon® membranes. Each membrane was subjected to the RASPE process in the presence of 0.2 pmole of p53 exon 8 target DNA. The signal intensity of primer extension products positive for p53 exon 8 DNA was similar at each location.

FIG. 3. Signal intensity of primer extension products positive for p53 exon 8 DNA respectively with 0.5 fmole and 0.1 fmole of p53 target DNA.

FIG. 4. Discrimination in the RASPE process of p53 target DNA by oligonucleotide specific therefor.

FIG. 5. Allele-specific discrimination by an oligonucleotide primer specific for the wild-type CF gene.

CLAIMS

1. A process for determination of at least one target nucleotide at at least one particular location within at least one polynucleotide sequence in a test sample, said target nucleotide(s) constituting a 5' terminal nucleotide of a target nucleotide sequence within said polynucleotide sequence(s), said process including the steps of:

(i) immobilizing one or more arrays of identical oligonucleotide primers with a solid support defining one or more reaction zones wherein each of said oligonucleotide primers in a respective array is capable of hybridizing with a corresponding target nucleotide sequence;

(ii) contacting the reaction zone with the test sample;

(iii) hybridizing at least one of said oligonucleotide primers with the polynucleotide sequence to form a hybrid wherein the polynucleotide sequence extends in a 3' to 5' direction beyond the 3' terminal nucleotide of the at least one oligonucleotide primer;

(iv) extending the at least one oligonucleotide primer of the hybrid beyond the 3' terminal nucleotide thereof in the 5' to 3' direction using the polynucleotide sequence as a template in the case when the 3' terminal nucleotide of the at least one oligonucleotide primer is complementary to the 5' terminal nucleotide of said target nucleotide sequence, said extension effected in the presence of a polymerization agent and nucleotide precursors to form a duplex including an extended primer molecule wherein at least one nucleotide incorporated into the extended primer molecule is a detectably-modified nucleotide;

(v) denaturing the duplex to free the polynucleotide sequence from the extended primer molecule;

(vi) carrying out steps (iii) to (v) one or more times; and

(vii) detecting presence of a signal associated with the detectably-modified nucleotide in the extended primer molecule at the reaction zone to effect said determination.

2. The process of claim 1 wherein the step of immobilizing (i) is

characterized in that a single array of identical oligonucleotide primers is immobilized at a single reaction zone of the solid support.

3. The process of claim 1 wherein the step of immobilizing (i) is characterized in that a plurality of distinct arrays of oligonucleotide primers are immobilized at discrete reaction zones of the solid support, wherein each of said reaction zones comprises a single array of identical oligonucleotide primers capable of determining a single target nucleotide under test.

4. The process of claims 1-3 further comprising, after step (vi), the step of separating unincorporated nucleotide precursors from the solid support.

5. The process of claim 4 wherein the separating step comprises washing from the solid support the unincorporated nucleotide precursors.

6. The process of claims 1-3 wherein the polymerization agent is a primer-dependent DNA polymerase having substantially no 3' exonuclease activity.

7. The process of claim 6 wherein said DNA polymerase is thermostable.

8. The process of claim 6 wherein the polynucleotide sequence in the test sample is DNA.

9. The process of claim 8 wherein the DNA is double-stranded and wherein, prior to the hybridizing step (iii), the DNA is denatured such that the DNA is substantially single-stranded.

10. The process of claim 6 wherein the nucleotide precursors comprise deoxynucleoside triphosphates.

11. The process of claim 10 wherein at least one of said deoxynucleoside triphosphates is a detectably-modified deoxynucleoside triphosphate.

12. The process of claim 6 wherein the nucleotide precursors comprise dideoxynucleoside triphosphates.

13. The process of claim 12 wherein at least one of said dideoxynucleoside triphosphates is a detectably-modified dideoxynucleoside triphosphate.

14. The process of claim 3 wherein the plurality of distinct arrays of oligonucleotides are capable of determining different alleles of a structural gene.

15. The process of claim 14 wherein said different alleles include at least one wild-type allele and at least one mutant allele.

16. The process of claim 3 wherein the plurality of distinct arrays of oligonucleotides are capable of determining different genetic loci.

17. The process of claim 16 wherein the genetic loci are associated with different inherited or acquired genetic disorders.

18. A solid support for determination of at least one target nucleotide at at least one particular location within at least one polynucleotide sequence in a test sample, said target nucleotide(s) constituting a 5' terminal nucleotide of a target nucleotide sequence within said polynucleotide sequence(s), said solid support comprising:

one or more arrays of identical oligonucleotide primers immobilized thereon defining one or more reaction zones, wherein each of said oligonucleotide primers in a respective array is capable of hybridizing with a corresponding target nucleotide sequence and has a 3' terminal nucleotide complementary to said target nucleotide to form a hybrid which permits extension of the oligonucleotide primer of the hybrid in the presence of a polymerization agent and nucleotide precursors.

19. The solid support of claim 18 wherein a single array of identical oligonucleotide primers is immobilized at a single reaction zone of the solid support.

20. The solid support of claim 18 wherein a plurality of distinct arrays of oligonucleotide primers are immobilized at discrete reaction zones of the solid support, wherein each of said reaction zones comprises a single array of identical oligonucleotide primers capable of determining a

single target nucleotide under test.

21. The solid support of claim 20 wherein the plurality of distinct arrays of oligonucleotides are capable of determining different alleles of a structural gene.

22. The solid support of claim 21 wherein said different alleles include at least one wild-type allele and at least one mutant allele.

23. The solid support of claim 20 wherein the plurality of distinct arrays of oligonucleotides are capable of determining different genetic loci.

24. The solid support of claim 23 wherein the genetic loci are associated with different inherited or acquired genetic disorders.

25. A kit for determination of at least one target nucleotide at at least one particular location within at least one polynucleotide sequence in a test sample, said target nucleotide(s) constituting a 5' terminal nucleotide of a target nucleotide sequence within said polynucleotide sequence(s), said kit comprising:

(a) one or more arrays of identical oligonucleotide primers immobilized on a solid support defining one or more reaction zones, wherein each of said oligonucleotide primers in a respective array is capable of hybridizing with a corresponding target nucleotide sequence and has a 3' terminal nucleotide complementary to said target nucleotide to form a hybrid which permits extension of the oligonucleotide primer of the hybrid in the presence of a polymerization agent and nucleotide precursors;

(b) a plurality of nucleotide precursors, wherein at least one of said nucleotide precursors is a detectably-modified nucleotide precursor;

(c) a polymerization agent capable of extending said oliginucleotide primers in a 5' to 3' direction when the 3' terminal nucleotide of the oligonucleotide primer is complementary to the target.

26. The kit of claim 25 wherein a single array of identical

oligonucleotide primers is immobilized at a single reaction zone of the solid support.

27. The kit of claim 25 wherein a plurality of distinct arrays of oligonucleotide primers are immobilized at discrete reaction zones of the solid support, wherein each of said reaction zones comprises a single array of identical oligonucleotide primers capable of determining a single target nucleotide under test.

28. The kit of claim 27 wherein the plurality of distinct arrays of oligonucleotides are capable of determining different alleles of a structural gene.

29. The kit of claim 28 wherein said different alleles include at least one wild-type allele and at least one mutant allele.

30. The kit of claim 27 wherein the plurality of distinct arrays of oligonucleotides are capable of determining different genetic loci.

31. The kit of claim 30 wherein the genetic loci are associated with different inherited or acquired genetic disorders.

nucleotide.

32. An apparatus for amplification and/or determination of a target nucleic acid, said apparatus comprising:

a reaction zone, in use, receiving a solid support having immobilized thereto first nucleic acids as well as receiving a reaction fluid for immersing therein said first nucleic acids, said reaction fluid comprising second nucleic acids being free in solution, wherein said target nucleic acid is included in said first nucleic acids or in said second nucleic acids;

a thermoregulation means for modulating the temperature of said reaction fluid at said reaction zone to facilitate at least in part said amplification and/or determination;

a denaturation zone for receiving said reaction fluid, and for denaturing said second nucleic acids, said denaturation zone being spaced from said reaction zone and in fluid communication therewith; and

means for moving said reaction fluid between said reaction

zone and said denaturation zone during the course of said amplification and/or determination.

33. The apparatus of claim 32 wherein the reaction zone is located within a reaction chamber.

34. The apparatus of claim 33 wherein internal surfaces of the reaction chamber are comprised of a hydrophobic material.

35. The apparatus of claim 33 wherein the reaction chamber comprises means for retaining said solid support therewithin.

36. The apparatus of claim 33 wherein the reaction chamber comprises means for introduction of said solid support therewithin and removal therefrom.

37. The apparatus of claim 32 wherein the thermoregulation means comprises a heat exchanger for modulating the temperature of the reaction fluid at the reaction zone.

38. The apparatus of claim 37 wherein the heat exchanger is a Peltier cell.

39. The apparatus of claim 32 wherein the thermoregulation means is microprocessor controlled to effect automatic sequential modulation of the reaction fluid temperature at the reaction zone.

40. The apparatus of claim 32 wherein the denaturation zone comprises a heat exchanger for heat denaturing said second nucleic acids.

41. The apparatus of claim 32 wherein the means for moving the reaction fluid between the reaction zone and the denaturation zone is effected by at least one pump.

42. The apparatus of claim 32 further comprising means for introducing the reaction fluid therein.

43. The apparatus of claim 42 wherein said means for introducing the reaction fluid comprises one or more ports in fluid communication with the apparatus.

44. The apparatus of claim 32 further comprising one or more

auxiliary vessels in fluid communication with the apparatus such that reagents for said amplification and/or determination may enter or exit the apparatus.

45. The apparatus of claim 32 further comprising a display means for displaying the temperature of the reaction zone.

46. The apparatus of claim 32 further comprising a detection means for detecting a probe nucleic acid or an extension product thereof attached to the solid support.

47. A process for amplification and/or determination of a target nucleic acid in a test sample suspected of containing said target nucleic acid, said method including the steps of:

(a) immobilizing first nucleic acids onto a solid support at a reaction zone;

(b) immersing said first nucleic acids in a reaction fluid comprising second nucleic acids being free in solution, wherein said target nucleic acid is included in said first nucleic acids or in said second nucleic acids if said target nucleic acid is present in said test sample;

(c) modulating the temperature of said reaction fluid at said reaction zone;

(d) denaturing said second nucleic acids at a denaturation zone being spaced from said reaction zone and in fluid communication therewith; and

(e) moving said reaction fluid between said reaction zone and said denaturation zone;

characterized in that said amplification and/or determination is effected after carrying out steps (c), (d) and (e) one or more times.

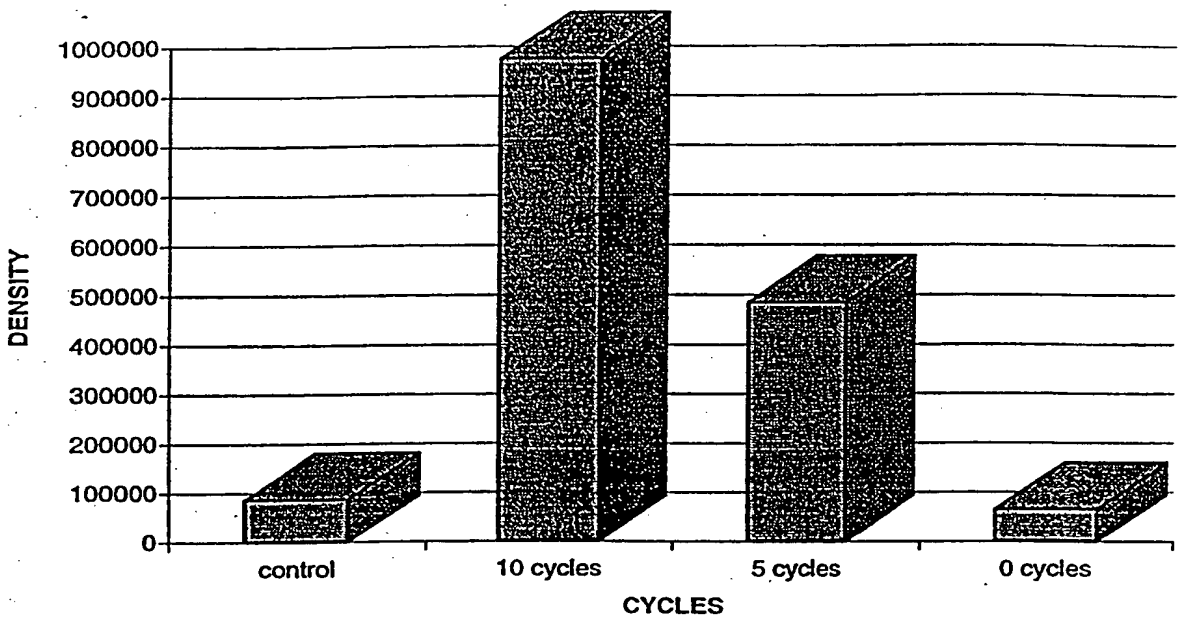
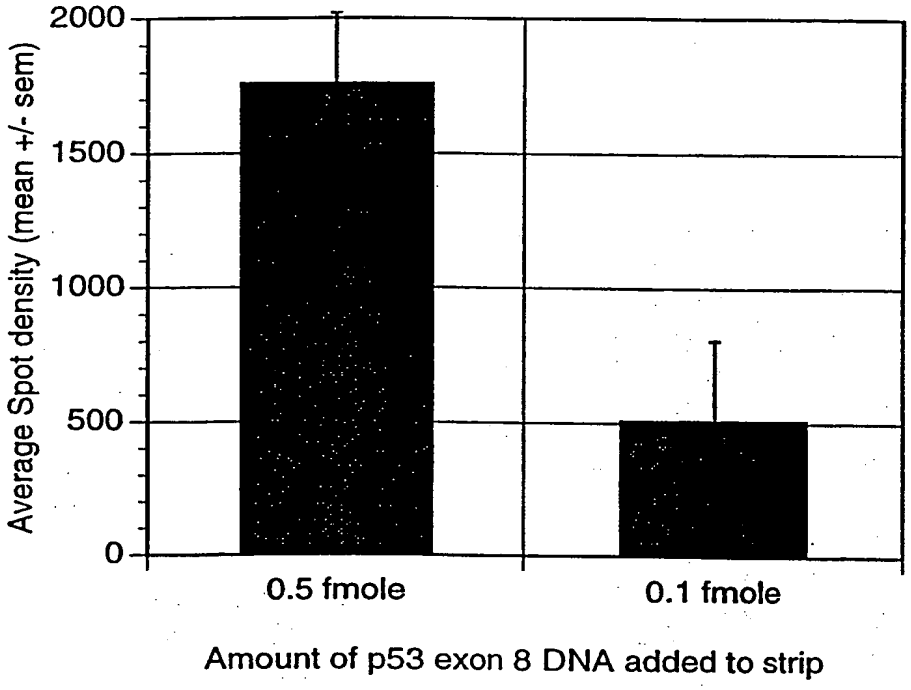

1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
DENSITY
control
10 cycles
5 cycles
0 cycles
CYCLES

FIG. 1

FIG. 2


2000
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1000
500
0
Average Spot density (mean +/- sem)
0.5 fmole
0.1 fmole
Amount of p53 exon 8 DNA added to strip

FIG. 3

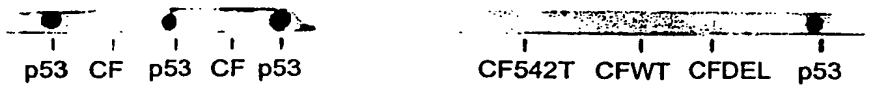

p53 CF p53 CF p53
CF542T CFWT CFDEL p53

FIG. 4

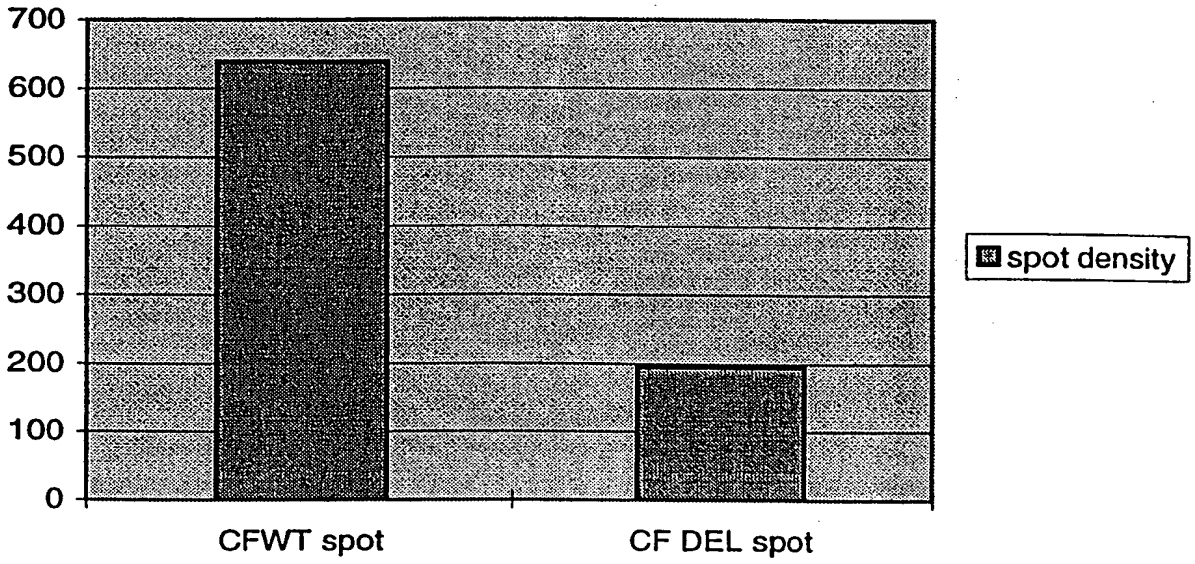

700
600
500
400
300
200
100
0
CFWT spot
CF DEL spot
spot density

FIG. 5

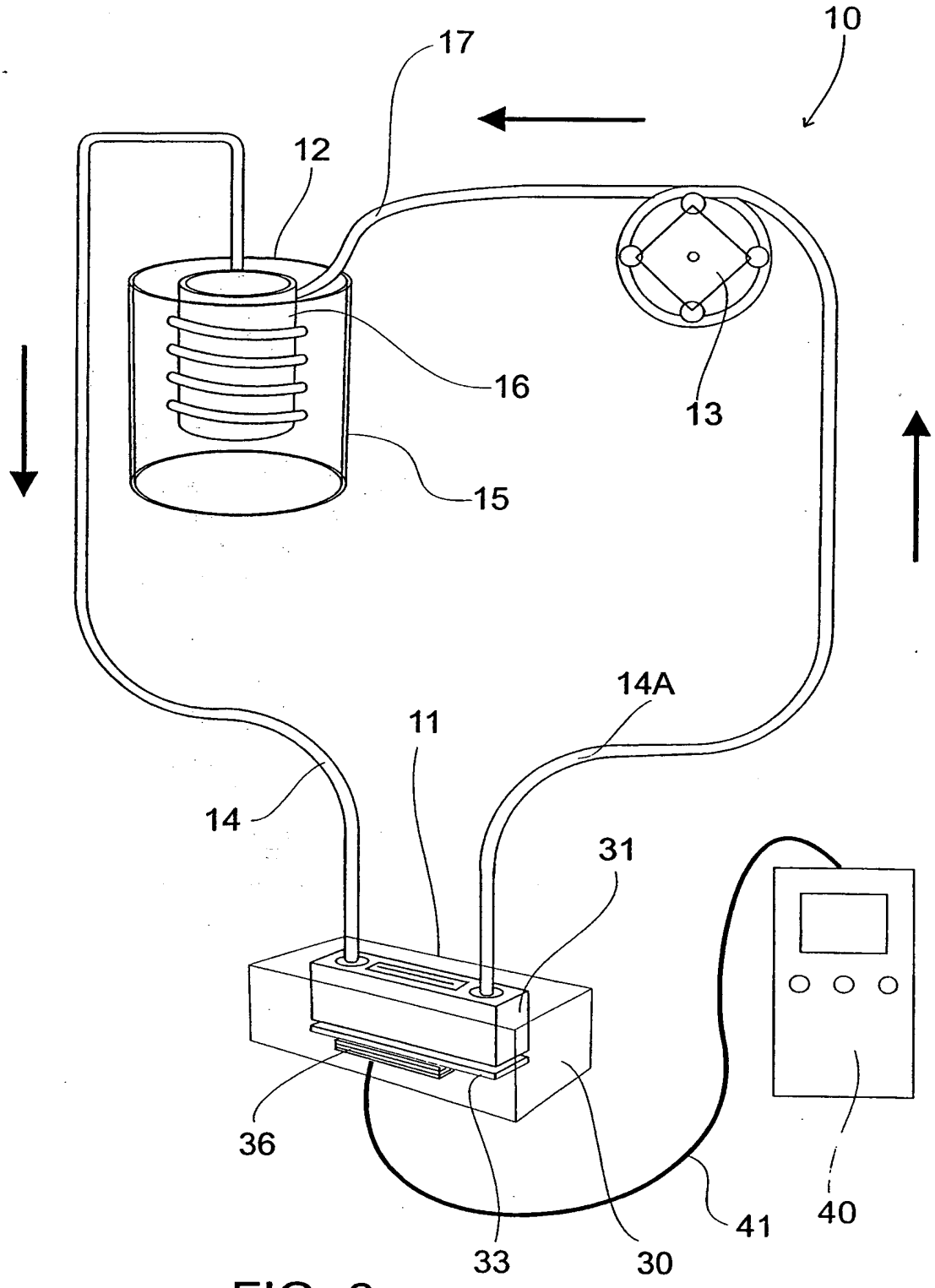


FIG. 6

FIG. 7

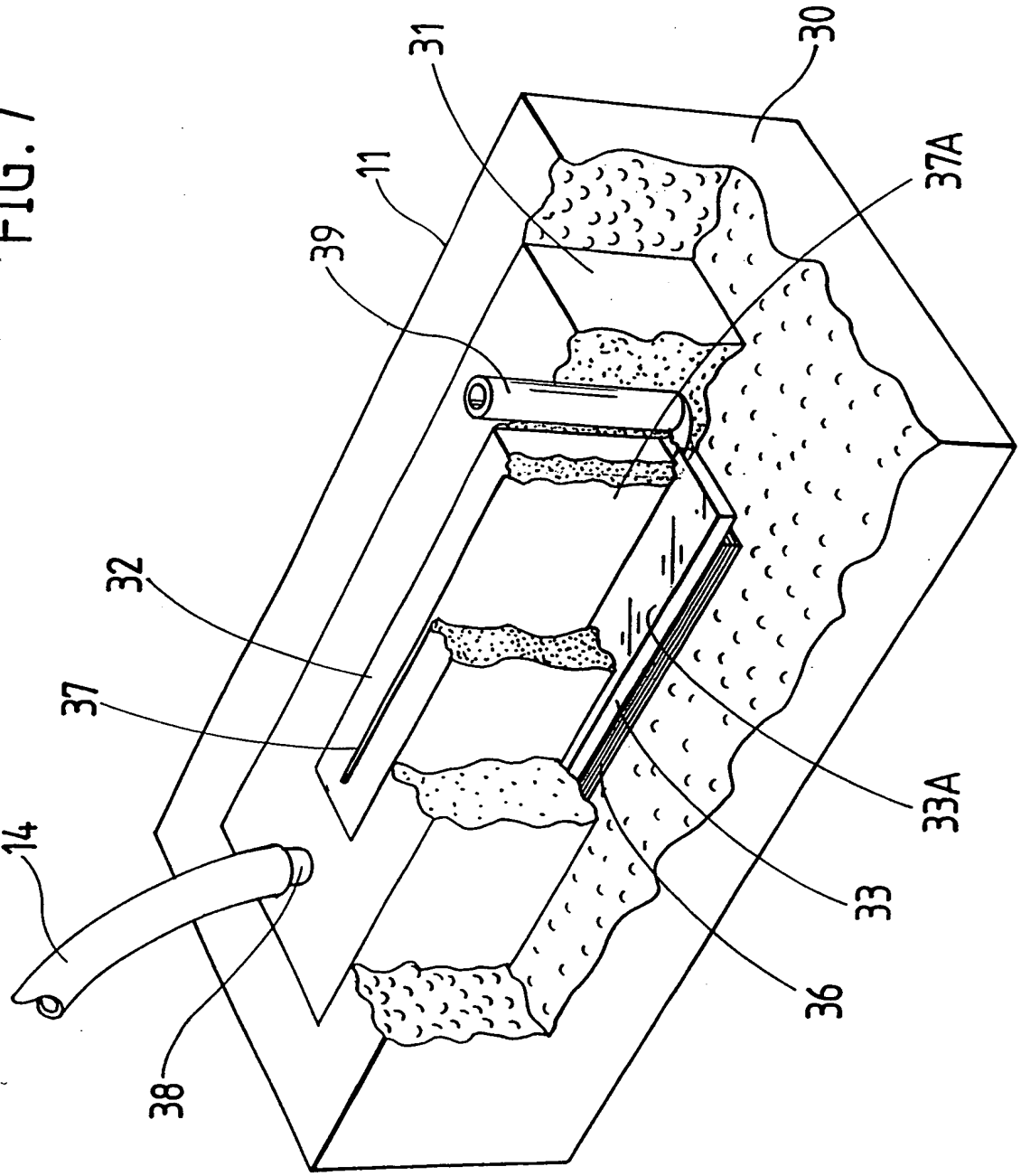


FIG. 8

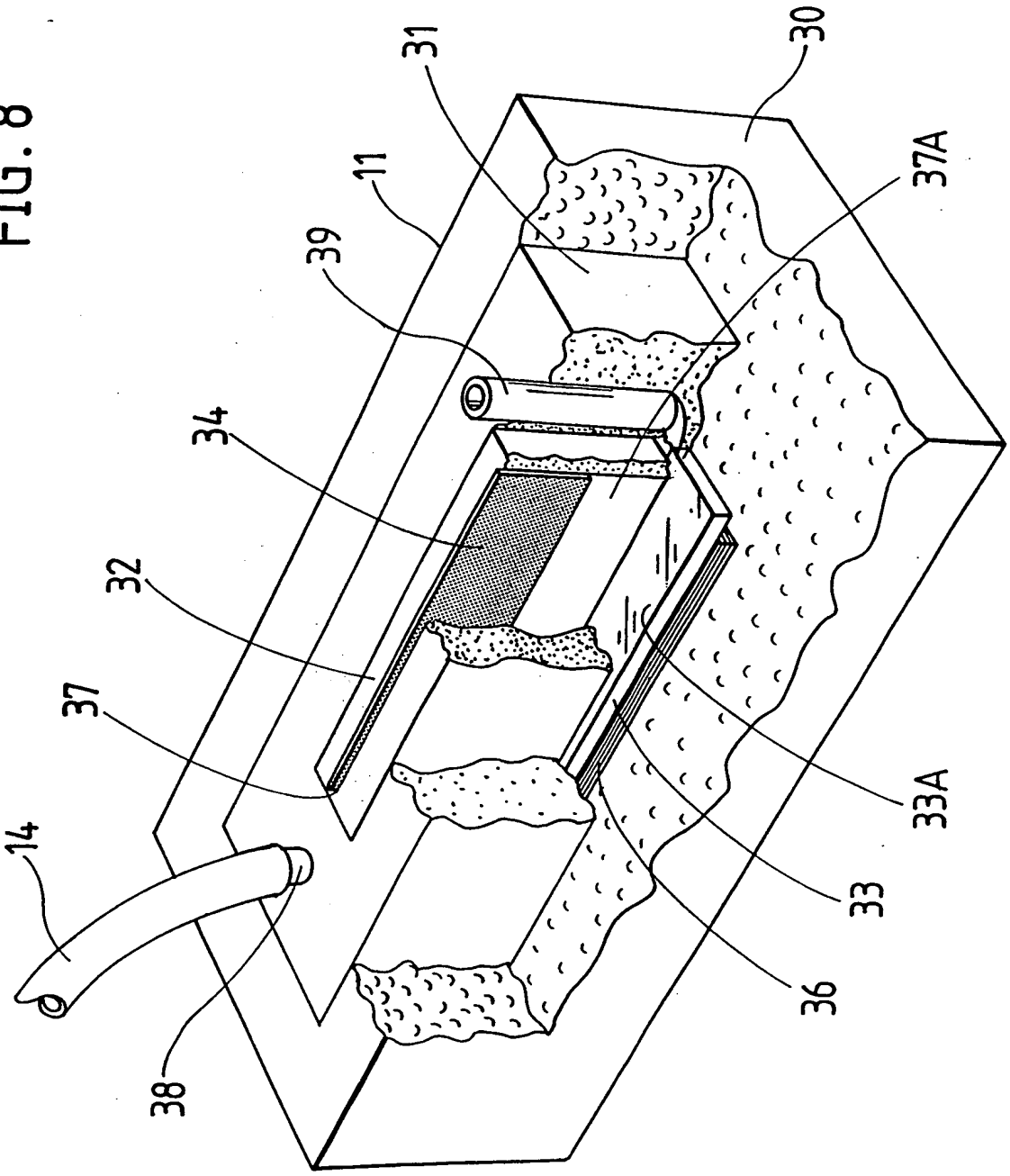
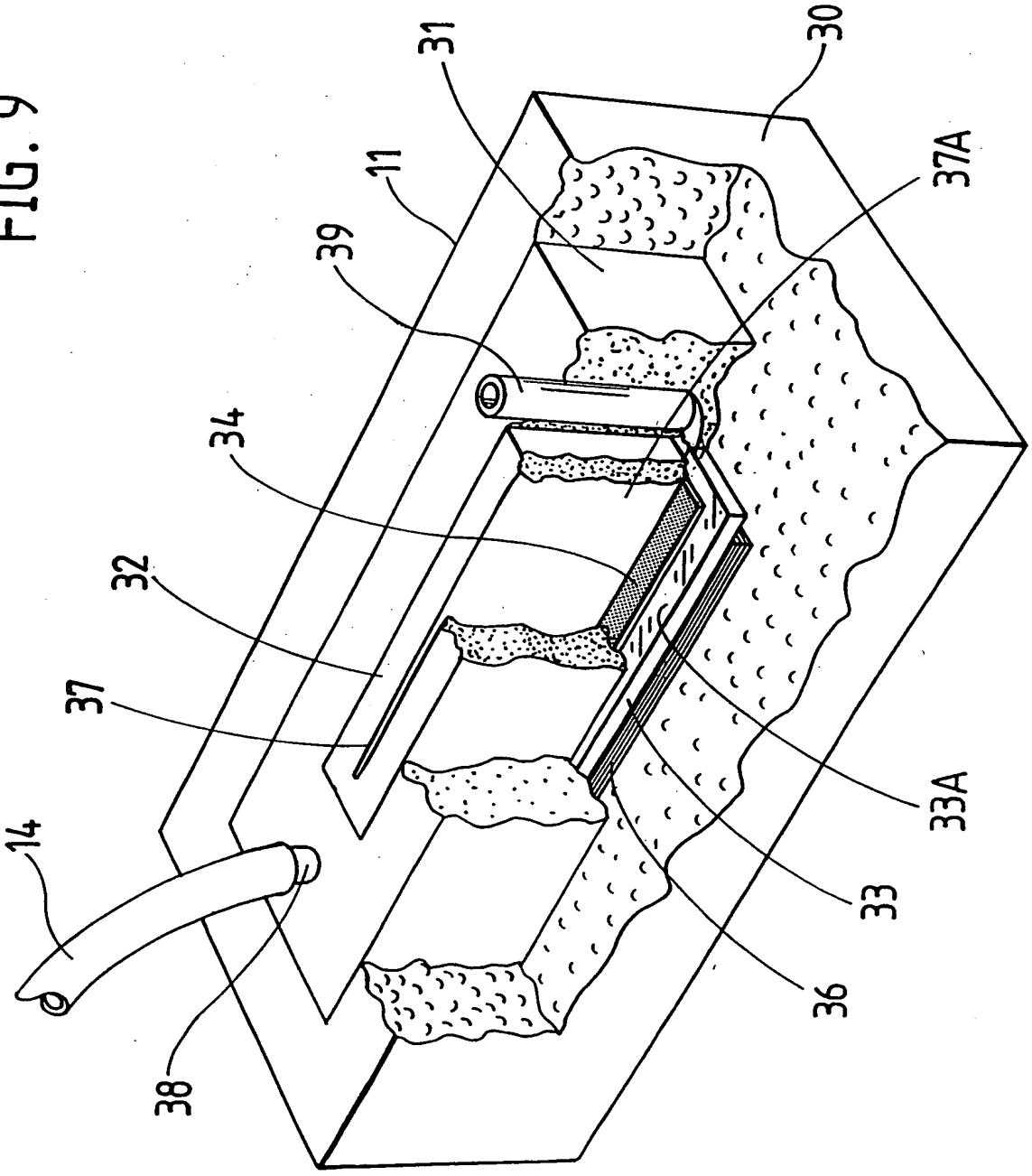


FIG. 9



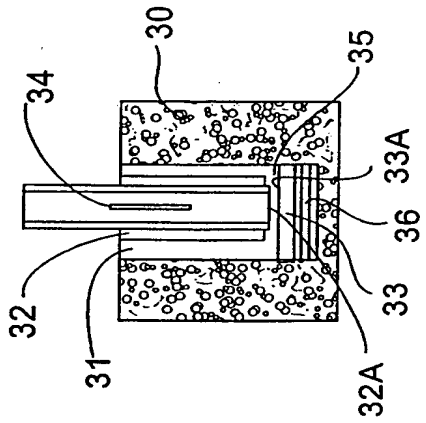


FIG. 11

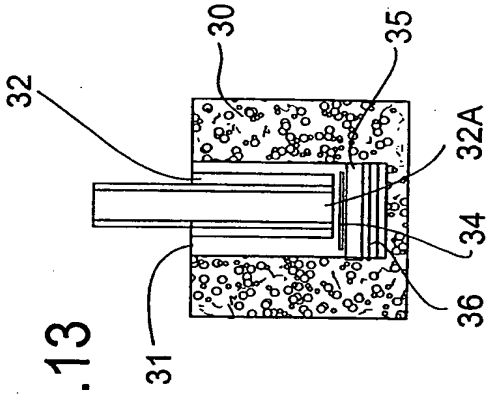


FIG. 13

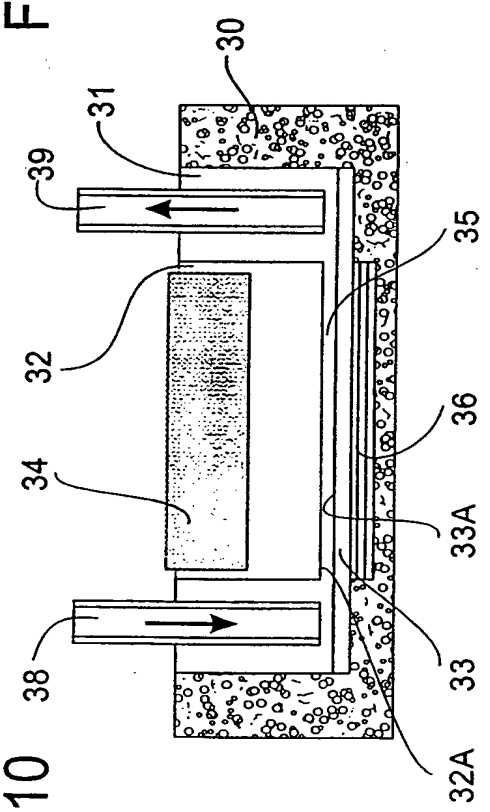


FIG. 10

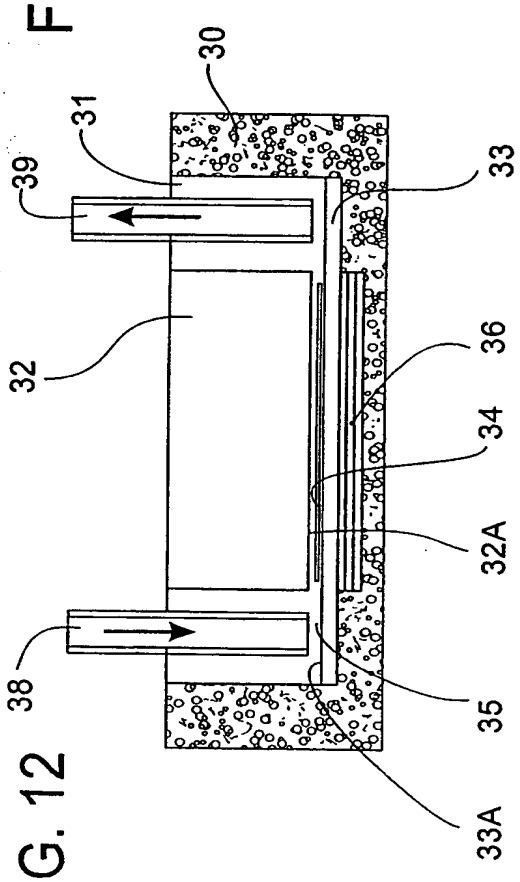


FIG. 12

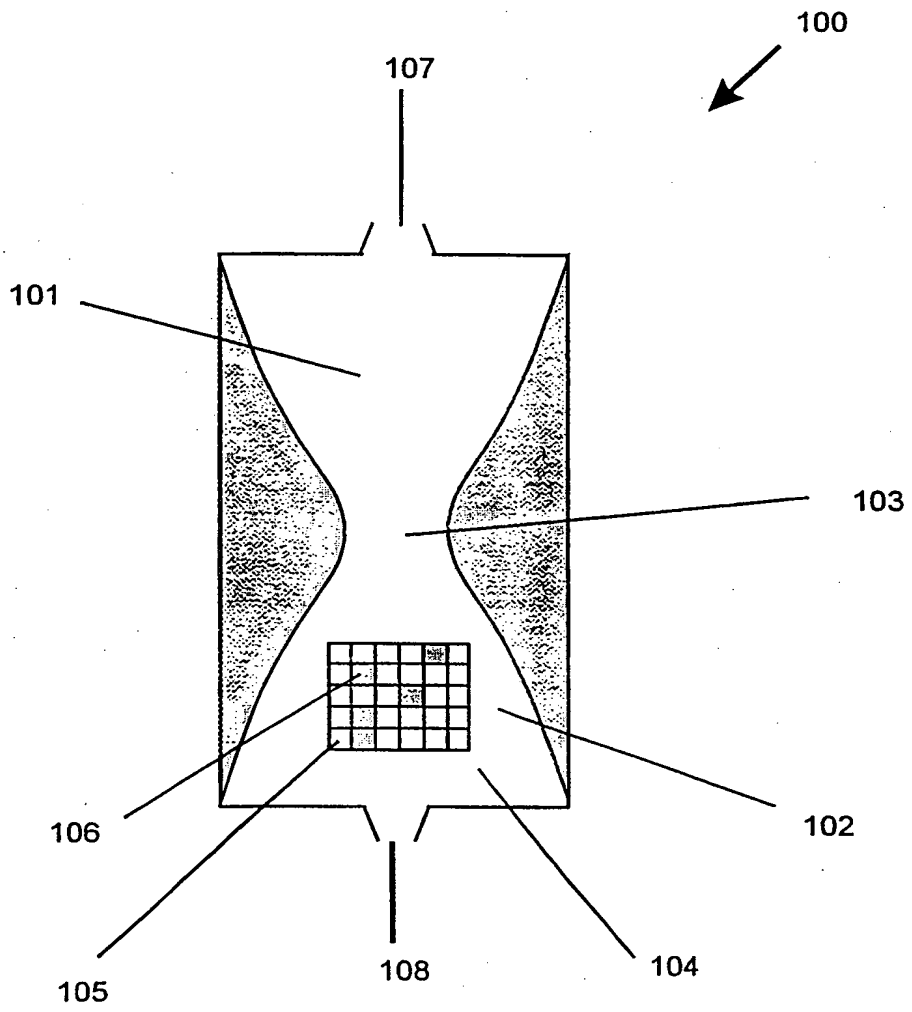


FIG. 14

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00875

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12Q 1/68, C12M 1/40

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q 1/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Orbit (WPAT): C12M and Immobili: and Denator; and
C12Q 1/68 and (support: or immobili:) and (termina: or residu:)
STN (CA, BIOSIS, MEDLINE): (primer# or oligonucleotide#) (10N) (immobil? or support?) and (mismatch? or match?)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96/31622 A (ISIS Innovation Limited) 10 October 1996 Whole document. In particular page 2 lines 27-30, page 7 lines 12-14, page 21 lines 8-21 and claim 1	1-31, 47
X	EP 478 319 A (Kabushiki Kaisha Toshiba) 1 April 1992 Figure 1, and page 14 lines 43-51	32-46

☐ Further documents are listed in the continuation of Box C

☒ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 February 1998	18 FEB 1998

Name and mailing address of the ISA/AU
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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00875

Box 1 Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 47
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Due to the broad nature of the claim, the search has been restricted to the one carried out for claims 1-31.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/AU 97/00875

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	96/31622	EP	820524	GB	9507238
EP	478319	DE	69125441	JP	5199898

END OF ANNEX